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Innate responses and biomarkers of resistance to *Eimeria* infection in the chicken.

Abi Bremner

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Author's Declaration

I declare that this thesis has been composed solely by myself, the work presented is entirely my own except where stated otherwise by reference or acknowledgement, and that it has not been submitted, in whole or in part, in any previous application for a degree or other professional qualification

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Abstract

Coccidiosis is an intestinal disease caused by the protozoan parasite *Eimeria*, of which *E. tenella* and *E. maxima* are a common cause of disease in the poultry industry, causing weight gain loss, decreased feed efficiency and mortality in poultry. Coccidiosis is usually controlled by the application of anti-coccidial drugs or by vaccination, but drug resistance in *Eimeria* has been reported and vaccines require the passage of live *Eimeria* oocysts through the birds and are therefore expensive and difficult to produce. Alternative solutions are to develop subunit vaccines and to breed chickens for resistance to *Eimeria* by identifying resistance biomarkers, both of which require characterisation of the chicken immune response to *Eimeria*. To characterise immune responses to *Eimeria*, this study aimed to investigate the response of antigen presenting cells (APC) to *Eimeria* and determine which chicken pathogen recognition receptors (PRRs) recognise *Eimeria* vaccine candidates using in vitro techniques. The role of T helper (Th) 17 cells during *E. maxima* and *E. tenella* infection was also investigated *in vivo* through infection of a commercial broiler line. This study also aimed to identify biomarkers of *Eimeria* resistance by characterising the immune response to *E. maxima* and *E. tenella* in chicken lines which exhibit differential resistance and susceptibility to both these *Eimeria* spp.

The development of chicken bone marrow-derived macrophage (BMM) and dendritic cell (BMDC) cultures provides an opportunity to study the responses of host-derived APC to *Eimeria* antigens and potential vaccine candidates in vitro. Here, both BMM and BMDC responded to an *E. tenella* oocyst crude lysate by upregulating mRNA expression of proinflammatory mediators (*IL1B*, *IL6* and

NOS2), BMM appeared more regulatory in nature (upregulated *IL10* mRNA expression) and BMDC appeared more Th1-promoting (upregulated *IFNG* mRNA expression). Immune mapped protein 1 (IMP1) and apical membrane antigen 1 (AMA1) are two *Eimeria* vaccine candidates that have been shown to elicit protective immunity to *Eimeria*. In response to vaccine candidates IMP1 and AMA1, BMM responded in an inflammatory fashion through increased expression of *IL6* and *NOS2* mRNA. These results indicate that chicken macrophages and dendritic cells can recognise *Eimeria* and *Eimeria* vaccine candidates and facilitate inflammation though production of proinflammatory cytokines, but also have roles in promoting Th1 responses and in immune regulation. In order to trigger innate immune responses, pathogen associated molecular patterns (PAMPs) must be recognised by host PRRs, present on the surface of APC. Currently it is not known which *Eimeria* PAMPs are detected by which chicken PRRs. Use of a reporter gene assay identified that recombinant IMP1 and AMA1 are recognised by Toll-like receptor (TLR)1LB/2A heterodimers however further investigation is needed to determine other *Eimeria* PAMPs that are recognised by other chicken PRRs.

Th1 responses are known to be important for the resolution of *Eimeria* infection however Th17 responses during *Eimeria* infection are less well characterised. Thought to contribute to immunopathology during *Eimeria* infection, Th17 responses represent a potential target in improving the outcome of *Eimeria* infection in chickens. Surprisingly, RT-qPCR analysis revealed no changes in the mRNA expression of Th17-associated cytokines in the gut of *E. maxima*- or *E. tenella*-infected Ross 308 broilers with the exception of *IL21*, indicating that IL-21 is acting in another capacity than as a Th17 effector during *Eimeria* infection. IL-21 is a

highly pluripotent cytokine and further study would be required to characterise the role of IL-21 during *Eimeria* infection. In order to breed chickens for resistance, biomarkers of *Eimeria* resistance must first be identified. Line 15I and C.B12 chickens display inverse resistance and susceptibility to *E. maxima* and *E. tenella*. To identify biomarkers of resistance to *E. maxima* and *E. tenella*, the immune response of these lines to both *Eimeria* spp. was phenotyped. A higher increase in serum IL-10 was observed in *E. maxima*-infected susceptible line 15I than line C.B12 supporting a previous report that IL-10 is involved in susceptibility to *E. maxima*. RT-qPCR analysis revealed earlier increases of *IFNG*, *IL10* and *IL21* mRNA in the gut of resistant line C.B12 birds following *E. maxima* infection, indicating that a prompt immune response is a factor in resistance to *E. maxima*. No biomarkers of resistance to primary *E. tenella* infection were identified and further interrogation of the immune responses of these lines is required, particularly in response to secondary *E. tenella* infection.

The results of this study have furthered our understanding of the role of APCs during *Eimeria* infection and following vaccination with IMP1 and AMA1 and support IMP1 and AMA1 as suitable vaccine candidates. IL-21 was identified as an important cytokine during *Eimeria* infection and further study is required to assess if IL-21 is beneficial or damaging to clearance of the parasite and to evaluate its potential as a therapeutic target. This study also confirmed previous findings that IL-10 is involved in susceptibility to *Eimeria* and identified that a rapid response is important for resistance to *E. maxima*, providing a basis for further study to identify biomarkers of *Eimeria* resistance.

Lay Summary

Coccidiosis is a disease of the gut caused by a parasite known as *Eimeria*. Two species, *E. tenella* and *E. maxima*, are a common cause of coccidiosis in the poultry industry, causing reduced weight gain, decreased feed efficiency and mortality in chickens. Coccidiosis is usually controlled by using anti-coccidial drugs or by vaccination, but there have been reports that *Eimeria* is becoming resistant to these drugs and current vaccines contain whole *Eimeria* parasites which are expensive and difficult to produce. Alternative strategies to combat *Eimeria* infection are to develop other vaccines consisting of specific *Eimeria* proteins or to select chickens which are more resistant to *Eimeria* infection for breeding.

Antigen presenting cells (APC) are involved in the recognition and destruction of pathogens and in helping further immune responses by producing cytokines, proteins which exert effects on other cells. Before an immune response can occur, the pathogen must first be recognised by the immune system. To do this, proteins present on pathogens are recognised by receptors present on host immune cells. An aim of this study is to investigate the responses of APC to *Eimeria* proteins that have potential to become vaccines and to identify which chicken receptors identify these proteins. The results of this study showed that APC responded to proteins that could be used as vaccines by producing inflammatory cytokines and that these proteins were recognised by specific receptors on APC. This shows that APC help cause inflammation during *Eimeria* infection and that the proteins tested are suitable for development as vaccines.

Another important role of APC is to guide the T helper (Th) cell response. Th cells are important to ensure that the immune system remembers pathogens so that if the infection reoccurs, the immune system can respond quickly. Th1 and Th2 cells are the two main types of Th cells. Th1 cells are involved in immunity to pathogens that invade host cells whereas Th2 cells are important for immunity to pathogens that do not invade host cells. Th1 cells are known to be more important in immunity to *Eimeria* than Th2 cells. Th17 cells are a new type of Th cell that have been discovered in mammals and are important for inflammation. This study aimed to find out if Th17 cells are involved in the immune response to *Eimeria* in the chicken. The results of this study showed that Th17 cells were not important during *E. maxima* or *E. tenella* infection.

Some types of chickens are more resistant to different species of *Eimeria* than others. It is important to identify why some chickens are more resistant than others so that these can be selected for breeding. The immune response to *Eimeria* of two types of chicken, one resistant to *E. maxima* and one to *E. tenella*, were studied. High levels of the IL-10 cytokine was observed in the blood of chickens susceptible to *E. maxima* which shows that high IL-10 levels result in susceptibility to *Eimeria*. It was also found that increases in other cytokines occurred more quickly in chickens resistant to *E. maxima* than in susceptible chickens which shows that the timing of the immune response is important to resistance. No indicators of resistance to *E. tenella* were found.

It is important to characterise the chicken immune response to *Eimeria* to identify potential targets for treatment for use within the poultry industry. This study has

helped us to understand the immune response to *Eimeria* in the chicken and provided a starting point to select resistant chickens for breeding.

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Abbreviations and Acronyms

°C	Degree Celsius
%	Percentage
µM	Micromolar
µl	Microlitre
µg	Microgram
A. simplex	*Anisakis simplex*
AEC	3-amino-9-ethylcarbazole
AMA1	Apical membrane antigen 1
APC	Antigen presenting cell
BALT	Bronchus-associated lymphoid tissue
BAFF	B cell activating factor
BCR	B cell receptor
BMDC	Bone marrow-derived dendritic cell
BMM	Bone marrow-derived macrophage
BSA	Bovine serum albumin
C. parvum	*Cryptosporidium parvum*
C. rodentium	*Citrobacter rodentium*
CALT	Conjunctiva-associated lymphoid tissue
CD	Cluster of differentiation
ch	Chicken
CLR	C-type lectin receptor
ConA	Concanavalin A
CpG-ODN	CpG Oligodeoxynucleotide
CSF	Colony stimulating factor
CT	Caecal tonsil
CTL	Cytotoxic T lymphocyte
dATP	Deoxyadenosine triphosphate
DEAE	Diethylaminoethyl cellulose
DC	Dendritic cell
DMEM	Dulbecco's modified eagle's medium

DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Nucleoside triphosphate
dpi	days post infection
DSS	Dextran sulphate sodium
DTT	Dithiothreitol
E. acervulina	*Eimeria acervulina*
E. arlongi	*Eimeria arlongi*
E. bovis	*Eimeria bovis*
E. brunetti	*Eimeria brunetti*
E. coli	*Escherichia coli*
E. falciformis	*Eimeria falciformis*
E. maxima	*Eimeria maxima*
E. mitis	*Eimeria mitis*
E. necatrix	*Eimeria necatrix*
E. praecox	*Eimeria praecox*
E. pragensis	*Eimeria pragensis*
E. tenella	*Eimeria tenella*
E. vermiformis	*Eimeria vermiformis*
EDTA	Ethylebediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EtAg	Eimeria tenella oocyst crude lysate
FACS	Fluorescence-activated cell sorting
FAE	Follicle-associated epithelium
FBS	Fetal bovine serum
fDC	Follicular dendritic cell
GALT	Gut-associated lymphoid tissue
GC	Germinal center
GPI	Glycosylophosphatidylinositol
H. meleagridis	*Histomonas meleagridis*
h	Hour
HET	Heterophil extracellular trap

hps	Hours post stimulation
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IBDV	Infectious bursal disease virus
IBV	Infectious bronchitis virus
ICC	Immunocytochemistry
IELs	Intraepithelial lymphocytes
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMP1	Immune mapped protein 1
iNOS	Inducible nitric oxide synthase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobase
kDa	Kilo dalton
L. donovani	*Leishmania donovani*
L. Major	*Leishmania major*
LB	Lysogeny broth
LBP	Lipopolysaccharide binding protein
LCMV	Lymphocytic choriomeningitis virus
LPL	Lamina propria lymphocytes
LPS	Lipopolysaccharide
M. synoviae	*Mycoplasma synoviae*
M. tuberculosis	*Mycobacterium tuberculosis*
MALT	Mucosa-associated lymphoid tissue
M cell	Microfold cell
MDV	Marek's disease virus
mg	Milligram
MHC	Major histocompatibility complex
min	Minute
MIP	Macrophage inflammatory protein
ml	Millilitre

MLN	Mesenteric lymph node
mM	Millimolar
ms	Millisecond
MMP	Matrix metalloproteinase
MRC	Mannose receptor
mRNA	Messenger ribonucleic acid
NARF	National avian research facility
NEAA	Non-essential amino acids
NET	Neutrophil extracellular trap
NDV	Newcastle disease virus
NK cell	Natural killer cell
NLR	NOD-like receptor
nM	Nanometer
NOD	Nucleotide binding oligomerisation domain
NKT cell	Natural killer T cell
OCT	Optimal cutting temperature
OD	Optical density
OvPrP	Ovine prion protein
P. chabaudi	*Plasmodium chabaudi*
P. falciparum	*Plasmodium falciparum*
PAMP	Pathogen-associated molecule pattern
PBL	Peripheral blood lymphocyte
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
pg	Picogram
PMN	Polymorphonuclear lymphocyte
PMSF	Phenylmethylsulphonyl fluoride
pmol	Picomoles
poly I:C	Polyinosinic:polycytidylic acid
PP	Peyer's patch
PRR	Pathogen recognition receptor

RAPD-SCAR	Random amplification of polymorphic DNA - sequenced characterised amplified region marker
RIN	RNA integrity number
RLR	RIGI-like receptor
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
RORγt	RAR-related orphan receptor gamma t
ROS	Reactive oxygen species
RPMI-1640	Rosewell Park Memorial Institute 1640
RT	Room temperature
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
s	Second
S. typhimurium	*Salmonella typhimurium*
SAG	Surface antigen
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEAP	Secreted alkaline phosphatase
SLE	Systemic lupus erythematosus
SOB	Super optimal broth
STAT	Signal transducer and activator of transcription
T. congolense	*Trypanosoma congolense*
T. cruzi	*Trypanosoma cruzi*
T. gondii	*Toxoplasma gondii*
Taq	*Thermus aquaticus*
Tbet	T box expressed in T cells
TCR	T cell receptor
Tfh	T follicular helper
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
Treg	T regulatory cell
V	Volt
X-gal	5-bromo-4-chloro-3-indolyl-D-galactopyranoside

Chapter 1 Introduction

1.1 Chicken welfare and the poultry industry

As the world's population continues to grow, pressure is increasing to maximise food production to meet demand. In addition, consumer demand for high welfare products has risen. The poultry industry is under pressure to maximise profit and productivity, maintain affordability of produce and meet consumer demand for high welfare, high quality meat. Disease presents one of the biggest challenges to the production of livestock and the poultry industry is vulnerable to a range of pathogens including *Eimeria*, avian influenza virus, infectious bursal disease virus (IBDV), infectious bronchitis virus (IBV), Marek's disease virus (MDV) and Newcastle disease virus (NDV). In addition, chickens can also be infected with organisms such as *Campylobacter* and *Salmonella*, both of which may be asymptomatic in chickens but cause significant disease and mortality in humans. With the use of antibiotics banned across Europe in 2006 (Cogliani *et al.*, 2011), there is a growing need to develop new strategies for disease control.

1.1.1 Current strategies for control of *Eimeria*

Coccidiosis is an intestinal disease caused by the protozoan parasite *Eimeria*. *Eimeria* infection is common in the poultry industry and results in reduced weight gain, decreased feed efficiency and increased mortality, costing commercial chicken producers in the UK alone an estimated £38 million per annum (Shirley *et al.*, 2007). Within the poultry industry, coccidiosis is widely controlled through chemoprophylaxis with anti-coccidial drugs (coccidiostats) however drug resistance in *Eimeria* has been reported (Yadav *et al.*, 2001; Arabkhazaeli *et al.*, 2013). To increase longevity, coccidiostats have been used in rotation to slow the development of *Eimeria* drug resistance (Clark *et al.*, 2017). The safety of chemical residues in

food-producing animals has also been questioned and both consumer and legislative pressure is increasing to reduce the use of coccidiostats in the poultry industry.

Alternatively, the use of live vaccines, using both non-attenuated and attenuated strains of parasite, have been employed to induce a low level of infection in commercial flocks leading to immune protection against *Eimeria.* Vaccines are produced through the passage of *Eimeria* oocysts through birds therefore their production is expensive and due to the high genetic diversity between different *Eimeria* spp., successful vaccines must be composed of multiple spp. of *Eimeria* to confer adequate protection in the field (Shirley *et al.*, 2005). Recombinant subunit and DNA vaccines are a potential alternative to the use of live vaccines, but again, high genetic diversity between and within *Eimeria* spp. means target antigens must be highly homologous across *Eimeria* spp. or vaccines must target multiple antigens (Clark *et al.*, 2017). Resistance and susceptibility to various *Eimeria* spp. has been demonstrated in different chicken lines (Long, 1968a; Bumstead *et al.*, 1995; Smith *et al.*, 2002), and an alternative strategy in combatting *Eimeria* would be to improve current vaccines or selectively breed chickens for resistance. Current strategies under investigation are the development of subunit vaccines (Blake *et al.*, 2011), DNA vaccines (Hoan *et al.*, 2014) and transgenic *Eimeria* vaccines where *Eimeria* of one spp. expresses antigenic peptides of another (Marugan-Hernandez *et al.*, 2017). Some attempts to breed chickens for *Eimeria* resistance have been successful; Rosenberg *et al.* (1954) first showed that it was possible to select birds which survived a high dose of *Eimeria* oocysts to produce resistant offspring, however resistance is lost after several generations. In addition, selection of birds for an inherently high inflammatory phenotype results in increased resistance to *E. tenella*

(Swaggerty *et al.*, 2015). However, due to the high diversity between *Eimeria* spp., a chicken line may be resistant to one spp., but susceptible to another (Bumstead *et al.*, 1992; Smith *et al.*, 2002), therefore there is a requirement to identify biomarkers of resistance to different *Eimeria* spp. which can then be applied for selection of resistant birds from a commercial population.

1.1.2 Life cycle of *Eimeria*

There are seven species of *Eimeria* that can infect chickens. The most frequently diagnosed *Eimeria* spp. in the poultry industry are *E. acervulina*, *E. tenella* and *E. maxima,* and chickens can be infected with multiple strains of *Eimeria* simultaneously (Long *et al.*, 1984). The other species of *Eimeria* that infect chickens are *E. necatrix*, *E. praecox*, *E. mitis* and *E. brunetti. E. tenella*, *E. necatrix* and *E. brunetti* cause haemorrhagic coccidiosis whereas *E. acervulina, E. maxima, E. praecox* and *E. mitis* cause malabsorptive coccidiosis (Long *et al.*, 1976).

The extracellular phase of the *Eimeria* life cycle begins when unsporulated oocysts (Figure 1-1A) are shed into the external environment in the host faeces. The oocyst sporulates by a process known as sporogeny where four sporocysts each containing two sporozoites develop (Figure 1-1B). Sporulated oocysts are ingested by the bird and taken into the gizzard where the oocyst wall breaks open and sporocysts are released. Sporocysts reach the gut and on contact with the bile salts, sporozoites burst out (Figure 1-1C; McDougald (1998)). The process by which sporozoites are released from the oocysts is known as excystation. Different *Eimeria* species are highly selective in both their choice of host species and site of infection within that host; *E. acervulina*, *E. maxima* and *E. tenella* infect the duodenum, jejunum and caecum respectively (Long *et al.*, 1976). Upon reaching the target site of infection

the sporozoites invade epithelial cells of the villi tips (Johnson *et al.*, 1970). Sporozoites then travel through the lamina propria to the crypt epithelium to undergo replication. Replication occurs by a process known as schizogony. The sporozoite forms a trophozoite that divides asexually to produce several merozoites, contained within a schizont (Figure 1-1D). Merozoites then lyse out of the epithelial cells and invade new cells, resulting in the formation of further schizonts containing more merozoites (Figure 1-1E). After a predetermined number of schizogony cycles (between two to four, dependent on the *Eimeria* spp.), a cycle of sexual replication occurs. Merozoites enter the process of gamogony whereby they invade epithelial cells and form micro- or macro-gamonts (Figure 1-1F). Those that are male divide multiple times to produce many biflagellate microgametes. Those that are female develop into one larger macrogamont. Microgametes are lysed from the host cell and invade cells containing macrogamonts. Macrogamonts become fertilised by the microgametes and a zygote is produced which then forms an oocyst wall, forming an unsporulated oocyst (Figure 1-1G) which is then shed in the faeces (Vetterling *et al.*, 1966; Warren *et al.*, 1967; McDougald, 1998).

During the *E. tenella* life cycle (reviewed by Chapman *et al*., 2003), sporozoites invade the caecal epithelium and travel through the lamina propria to invade the crypt epithelium, where first generation schizonts form (first observed from 48 h). Resulting merozoites invade further crypt epithelial cells, which migrate through the basement membrane into the connective tissue, forming second generation schizonts (observed from 84 h) which cause major haemorrhagic pathology. Resulting merozoites then invade epithelial cells at the tips of the caecal folds where third generation schizonts are formed (from 108 h). Third generation merozoites then

invade further epithelial cells, giving rise to gametocytes (from 114 h). *E. tenella* oocysts first appear in the faeces from 132 h post infection, lasting until 14 days post infection, with peak production at day 8. During *E. maxima* infection, sporozoites invade epithelial cells of the jejunum, travel through the lamina propria and invade crypt epithelial cells, forming first generation schizonts (appearing between 24 to 48 h). Merozoites are released producing second (48 to 72 h) and third (72 to 96 h) generation schizonts in the epithelium of the crypt openings and villi. Gamonts are observed in the villi epithelium and deeper tissue (96 to 120 h) and is the stage of the *E. maxima* life cycle which gives rise to pathology. *E. maxima* oocysts first appear in the faeces from 138 h after infection, with peak oocyst production at day 6 (Long, 1959; Millard *et al*., 1972; Rose *et al.,* 1976; Jenkins *et al*., 2017).

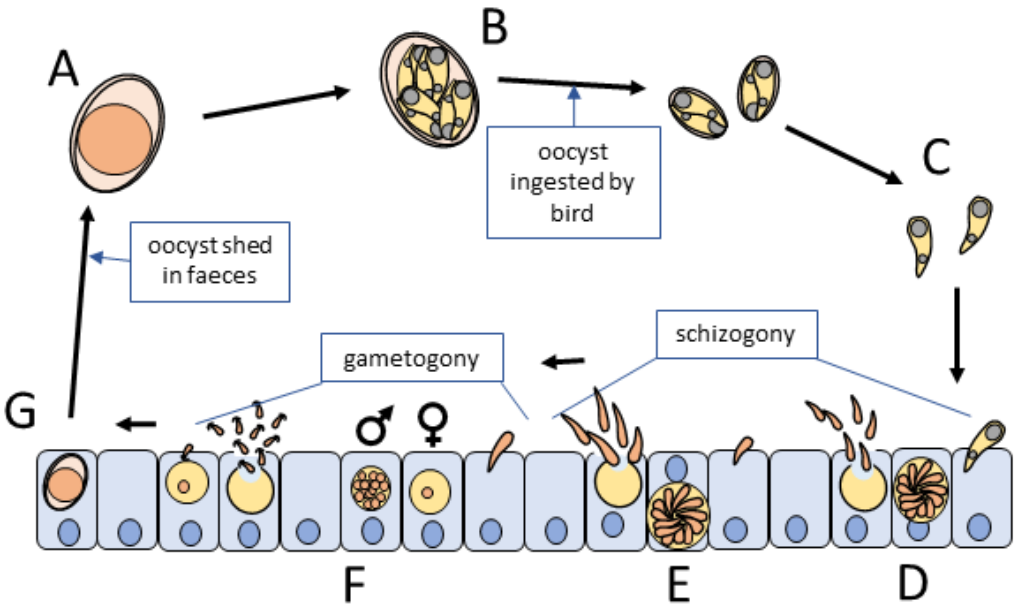

Figure 1-1: The *Eimeria* life cycle.

Little is known of the mechanism by which different *Eimeria* species recognise their specific infection sites. Vervelde *et al.* (1993b) identified the epitope E. TEN 11M-2 present on both the chicken caecal epithelium and *E. tenella* sporozoites but the mechanism by which *E. tenella* and other *Eimeria* species are attracted or attach to their specific sites of infection is unknown. It has also been shown that *E. maxima*, *E. acervulina* and *E. praecox* inoculated intra-caecally migrate back and cause infection in the small intestine and low numbers of *E. praecox* sporozoites inoculated intra-cloacally reached the small intestine (Long *et al.*, 1976) therefore the specificity to infection site of *Eimeria* spp. is unlikely due to the length of time between ingestion and sporozoite release.

1.2 Overview of the immune system

The immune system can be simplified into two main arms; the innate and the adaptive immune response. The innate response is the first line of defence against pathogens and comprised of physical barriers (i.e. skin), innate cells (i.e. natural killer (NK) cells), antimicrobial peptides (i.e. β-defensins, cathelicidins, the complement system) and the inflammatory response. The adaptive response is mounted following the innate response, is pathogen-specific and responsible for the rapid clearance of pathogens upon secondary exposure (Avian Immunology, 2nd Edition, Chapter 7). Adaptive immunity is comprised of cell-mediated (T cell - mediated) and humoral (B cell- and antibody-mediated) immunity. When the immune system encounters a pathogen, it is sensed via germline encoded pathogen recognition receptors (PRRs) present on the surface of many cell types including antigen presenting cells (APC) such as macrophages and dendritic cells (DCs), epithelial cells and NK cells. To initiate an adaptive response, APC present antigenic

peptides to naïve T cells and, depending on the cytokines produced, encourage their development into different T helper (Th) subtypes as detailed in section 1.3.2.1, thus driving cell–mediated and humoral adaptive responses (Avian Immunology, 2nd Edition, Chapter 10).

Lymphocytes are produced by and are able to relocate throughout the body via the lymphatic system consisting of lymphatic vessels, lymphoid organs and tissues and encapsulated lymph nodes. While lymph nodes have been described in avian spp., they do not possess encapsulated draining lymph nodes as mammals do (Biggs, 1957). Chicken lymphoid organs include the bone marrow, thymus and the Bursa of Fabricius and peripheral lymphoid tissues include the Harderian gland, spleen, mucosa-associated lymphoid tissue (MALT), Meckel's diverticulum (MD; the remnant of the yolk sac found halfway along the jejunum), caecal tonsils (CT) and Peyer's patches (PP). Haematopoetic stem cells from the bone marrow give rise to lymphoid and myeloid cells. Immature T cells migrate to the thymus to mature, similarly as in mammals, and immature B cells migrate to the Bursa of Fabricius, located at the cloaca, for maturation, occurring in the bone marrow in mammals (Avian Immunology, 2nd Edition, Chapter 3). The MALT includes gut-associated lymphoid tissue (GALT; described in section 1.4), conjunctiva-associated lymphoid tissue (CALT) and bronchus-associated lymphoid tissue (BALT; Avian Immunology, 2nd Edition, Chapter 2).

1.3 Innate immunity

1.3.1 Innate cells

Chicken innate cells include macrophages, DCs, NK cells, thrombocytes, mast cells and heterophils (the functional equivalent of mammalian neutrophils; Petrone *et al.*

(2002)). Macrophages are one of the most important innate cells and are vital in recognising and responding to pathogens, but also have roles in tissue development, repair and homeostasis. Tissue-resident macrophages recognise pathogens via PRRs (described in section 1.3.1.1) and mediate pathogen-killing via phagocytosis and formation of the phagolysosome (Qureshi, 1998) and through oxidative burst, whereby reactive oxygen species (ROS; including peroxide, hydroxyl radicals and superoxides) and reactive nitrogen species (RNS; including nitric oxide and superoxides) are released (Sung *et al.*, 1991; Qureshi *et al.*, 1994). In addition, macrophages can aid in inflammation and adaptive T cell responses through the production of pro-inflammatory cytokines (such as interleukin (IL)-1β and IL-6) and chemokines (such as macrophage inflammatory protein (MIP)1 and MIP2) but also in immune regulation through production of anti-inflammatory cytokines such as IL-10 (Klasing, 1998; Wu *et al.*, 2016). Although mainly considered an innate cell type, macrophages are also effectors of the adaptive immune response. Interferon (IFN)γ, produced as a component of the inflammatory response and as an effector cytokine during a Th1-polarised response (section 1.3.2.1) increases phagocytosis (Wang *et al.*, 2015), production of ROS and nitric oxide synthase (NOS) and cytokine production (He *et al.*, 2011) by macrophages. Macrophages also possess Fc receptors and complement receptors for the recognition of antibody and complement opsonised pathogens respectively (Dietert *et al.*, 1991). Additionally, macrophages polarised towards an M1 or M2 phenotype have been described in mammals (Mills *et al.*, 2000), although their presence has not yet been established in the chicken. M1 macrophages are considered important to inhibition of the pathogen, produce proinflammatory cytokines and are driven by IFNγ. M2 macrophages are important

to resolution of inflammation, tissue repair, exhibit enhanced phagocytosis and production of anti-inflammatory mediators such as IL-10 (Mills *et al.*, 2000).

Although macrophages also have the ability to present antigens to naïve T cells, DCs are considered professional APCs and act as an important bridge between innate and adaptive immunity. Immature DCs reside in tissues and upon recognition of pathogens via PRRs, migrate to areas containing naïve T cells where, following maturation, they can present pathogenic antigens via MHC class II molecules to $CD4^+$ T cells alongside co-stimulation with CD80/CD86 expressed on APCs with CD28 on T cells (Young *et al.*, 1994; Wu *et al.*, 2010). It is not precisely known where antigen presentation to naïve T cells occurs in chickens, however in mammals, this occurs in the lymph nodes (Ingulli *et al.*, 1997). DCs produce cytokines in response to pathogens, resulting in polarisation of Th responses (section 1.3.2.1). DCs are highly migratory; immature DCs express chemokine receptors CCR6 and CCR7 and upon maturation, down-regulate CCR6 and upregulate CCR7 expression *in vitro* (Wu *et al.*, 2011).

Heterophils are the main type of granulocyte in chicken blood and migrate towards sites of inflammation where they release proinflammatory cytokines and chemokines (Kogut *et al.*, 2005b). Heterophils, like neutrophils, are highly phagocytic and are able to undergo degranulation and oxidative burst (Kogut *et al.*, 1994). In contrast to mammalian neutrophils, chicken heterophils do not produce myeloperoxidase and therefore do not kill pathogens by oxidative burst as readily as neutrophils (Penniall *et al.*, 1975). Chicken heterophils are also capable of heterophil extracellular trap

(HET) formation in response to pathogens, whereby intracellular contents are released, trapping and killing pathogens (Chuammitri *et al.*, 2009).

In both birds and mammals, NK cells are large lymphocytes containing dense granules (Göbel *et al.*, 1994) and are found in the intestinal epithelium and peripheral blood (Göbel *et al.*, 2001). NK cell functions include cytotoxic activity and the release of cytokines and chemokines and in particular, NK cells respond to and produce IFNγ (Merlino *et al.*, 2002). Intestinal intraepithelial leukocytes (IELs) have also been shown to exhibit NK cell activity towards NK target cells, LSCC-RP9 (Göbel *et al.*, 2001). It has been shown that mammalian NK cells can respond to $MHCI^-$ cells, cells expressing pathogen encoded molecules and cells with up-regulated expression of self-molecules (Ljunggren *et al.*, 1990). Mammalian NK cells exhibit cytotoxic activity by the release of cytotoxic mediators such as perforin and granzyme, resulting in non-specific lysis of target and host cells and transcripts of chicken perforin and granzyme A are upregulated following *E. tenella* and IBDV infection (Rauf *et al.*, 2011; Wattrang *et al.*, 2016a). In addition, mammals also have NKT cells, cells that share both NK and T cell properties (Godfrey *et al.*, 2010) although the presence of NKT cells has not yet been confirmed in the chicken.

Avian thrombocytes are different to mammalian thrombocytes in that they are nucleated (Janzarik *et al.*, 1979). In birds and mammals, thrombocytes are important to haemostasis, production of cytokines (IL-10 and transforming growth factor (TGF)-β4 and upon stimulation with lipopolysaccharide (LPS), IL-1β, IL-6 and IL-12α (Ferdous *et al.*, 2008; Scott *et al.*, 2008; Paul *et al.*, 2012)) and chemokines (MIP1β; Lam (2002)) and wound healing (Wachowicz *et al.*, 1981). Avian

thrombocytes express Toll-like receptors (TLRs), CD80, CD86 and CD40 (Tregaskes *et al.*, 2005; Paul *et al.*, 2012), indicating that they may be involved in antigen presentation.

Granulocytes also include mast cells, basophils and eosinophils. Chickens possess fewer of these cells than mammals (Avian Immunology, 2nd Edition, Chapter 20). In chicken and mammals, mast cells are most abundant in tissues that border the external environment (Wight, 1970). Chicken mast cells are not as well characterised as their mammalian counterparts, however histamine and serotonin-containing granules have been identified in avian mast cells (Rose *et al.*, 1980). While mostly associated with allergy in mammals, mast cells are also important to wound healing, immune tolerance and immune responses to bacterial and parasitic infections (Kaliner, 1987), can produce cytokines and ROS, and are crucial to a rapid response to reinfection during acquired immunity through their recognition of Ag-specific immunoglobulin (Ig)E via Fc receptors present on the cell surface (Galli *et al.*, 2005). Although functional eosinophils have not yet been formally identified in the chicken, polymorphonuclear granulocytes (PMNs) with granules that readily stain with eosin have been identified (Maxwell, 1987). Eotaxins, a chemoattractant for mammalian eosinophils, and the eotaxin receptor have not been identified in the chicken genome. Mammalian eosinophils are granulocytes which circulate within the blood and function against multicellular parasites, such as Helminths, by releasing chemical mediators such as histamine, ROS and proinflammatory cytokines and, as are mast cells and basophils, implicated in allergy (reviewed by Rosenberg *et al.* (2013)). Chicken basophils are found circulating in the blood and are similar to mammalian basophils in that they are phagocytic (Dhodapkar *et al.*, 1982).

Mammalian basophils are similar to mammalian eosinophils in that they are able to produce histamine and bind IgE-opsonised pathogens (Stone *et al.*, 2010).

Epithelial cells are also important during innate immunity. Epithelial cells, particularly those lining mucosal tissues, are among the first cells pathogens encounter and are important in pathogen recognition and facilitating immune responses. Epithelial cells express PRRs on their surface (Iqbal *et al.*, 2005; Zhang *et al.*, 2012) and are able to respond to pathogens through production of cytokines (Li *et al.*, 2008), chemokines (Li *et al.*, 2009) which stimulate and attract cells including macrophages and heterophils, and anti-microbial peptides including β-defensins which mediate direct microbicidal activity (Zhao *et al.*, 2001; Zhang, L. *et al.*, 2016).

1.3.1.1 Pathogen Recognition Receptors

The chicken immune system is able to recognise pathogen-associated molecular patterns (PAMPs) via PRRs present on many cells including DCs, heterophils, macrophages, B cells and epithelial cells (He *et al.*, 2003; Kogut *et al.*, 2005a). Different PRRs are specialised to recognise different types of PAMPs which recruit different signalling cascades and transcription factors in order to initiate an appropriate immune response to that pathogen. The TLR family constitutes the major family of PRRs in both chickens and mammals; a comparison between the chicken and mammalian TLR families is given in Table 1-1. Chicken TLR1LA, TLR1LB, TLR2A and TLR2B are the functional homologs of the mammalian TLR1/6/10 family. In mammals, TLR1, 6 and 10 can form heterodimers with TLR2, whereas in chickens, TLR1LA and 1LB can form heterodimers with TLR2A or 2B. Heterodimers formed between TLR1LA or LB and TLR2A or B traditionally

recognise lipoproteins and peptidoglycans. Chicken TLR1A/2B heterodimers recognise diacylated and triacylated lipoproteins, recognised by TLR1/2, TLR2/10 and TLR2/6 heterodimers in mammals; chicken TLR1LB/2A heterodimers recognise peptidoglycan, recognised by TLR2 in mammals (Higuchi *et al.*, 2008). TLR4 in chickens and mammals recognises LPS, a common component on many bacterial surfaces, and is a crucial component in the LPS-receptor complex along with CD14, LPS binding protein (LBP) and myeloid differentiation factor (MD) 2 (Lizundia *et al.*, 2008). Chicken CD14 is a transmembrane protein (Wu *et al.*, 2009) whereas mammalian CD14 is a glycosylphosphatidylinositol (GPI)-anchored protein which directly binds LPS (Dil *et al.*, 2002). Chicken TLR21 is the equivalent of TLR9 in mammals and recognises CpG deoxyribonucleic acid (DNA) motifs (Brownlie *et al.*, 2009). TLR15 appears to be unique to avian and reptilian species. Reported ligands of TLR15 are whole yeast lysates (Boyd *et al.*, 2012) and diacylated lipopeptide from *Mycoplasma synoviae* (Oven *et al.*, 2013).

PRRs outwith the TLR family include C-type lectins (CLRs), RIG-I-like receptors (RLRs) and Nod-like receptors (NLRs). Membrane-bound surface PRRs recognise microbial PAMPs such as LPS and intracellular vesicle membrane-bound PRRs recognise vesicle-trapped PAMPs such as viral ribonucleic acid (RNA). There are also soluble cytosolic PRRs, including RLRs and NLRs. RLRs include retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). As in mammals, chicken MDA5 is important to the production of type 1 IFNs (IFNα and IFNβ) in response to polyinosinic:polycytidylic acid (poly I:C; Karpala *et al.* (2011)) and chicken LGP2 positively regulates MDA5 signalling (Liniger *et al.*, 2012). RLRs are DNA helicases

and recognise viral RNA present in the cytoplasm. RIG-I is present in avian species such as the duck and zebra finch but has not been identified in the chicken (Barber *et al.*, 2010). NLRs are cytosolic mammalian PRRs. NODs (nucleotide-binding oligomerisation domain) have not yet been described in the chicken but the NOD-1 sequence has been identified in the chicken genome and chickens do have NLRs (Lian *et al.*, 2012). In mammals, NOD-1 and NOD-2 bind peptidoglycan breakdown products of bacteria (Girardin *et al.*, 2003). C-type lectins including galectins, Ca^{2+}-dependent CLRs, siglecs and collectins are a family of carbohydrate recognition receptors which are all found in the chicken (Holmskov *et al.*, 1993; Angata *et al.*, 2007; Ruiz *et al.*, 2013). Galectins are secreted, most C-type lectins are membrane-bound and all siglecs are membrane-bound. C-type lectins recognise glycans, present on pathogens (reviewed by van Kooyk & Rabinovich, 2008).

Table 1-1: Comparison of mammalian and chicken TLRs and their ligands. HSP; Heat Shock Proteins.

TLR Family	Mammalian TLRs	Ligand	Chicken TLRs	Ligand	Cellular Localisation
TLR1/2	TLR1/6/10	lipoproteins, aribinomannan, peptidoglycan, fungal zymosan, GPI anchors, lipoteichoic acid, glycolipids, viral envelope proteins, mammalian HSP and heparin sulfate	TLR1LA & TLR1LB	lipoproteins and peptidoglycans	cell surface
	TLR2		TLR2A &TLR2B		
	TLR1 & 6 can form heterodimers with TLR2	bacterial peptidoglycan, diacy- (TLR1) and triacyl-(TLR6) peptides	TLR1s and TLR2 can form heterodimers		
TLR3	TLR3	viral dsRNA	TLR3	viral dsRNA	endosome
TLR4	TLR4 (in complex with MD2, LBP & CD14)	lipopolysaccaride (LPS), fungal mannan, glycoinositolphospholipids, viral envelope proteins, mammalian HSP and heparin sulfate	TLR4 (forms a complex with MD2, LBP and CD14)	lipopolysaccharide (LPS)	cell surface
TLR5	TLR5	flagellin	TLR5	flagellin	cell surface
TLR7	TLR7	viral ss RNA	TLR7	viral ssRNA, sythetic ssRNA	endosome
	TLR8	viral ssRNA	TLR8 (present as a pseudogene)	viral sRNA	
	TLR9	unmethylated CpG motifs, *Plasmodium* hemozoin	absent	-	
TLR15	absent	-	TLR15	whole yeast cell lysate, *M. synoviae* diacylated lipopeptide	cell surface
TLR21	absent	-	TLR21	unmethylated CpG motifs	endosome

1.1 Adaptive immunity

1.1.1 Cell-mediated immunity

The type of pathogen encountered by the immune system will define its response. In both chickens and mammals there are various Th cell subsets responsible for responding to different types of pathogen and maintaining tolerance to self-antigens. Peptides are presented via the major histocompatibility complex (MHC) to the T cell receptor (TCR), present on T cells. MHC class I molecules are located on the majority of cell types in the body and present host peptides to help maintain immune tolerance or peptides from intracellular pathogens. MHC class II molecules are mainly located on APC, heterophils and epithelial cells and present antigens from phagocytosed pathogens. CD8 and CD4 (for antigens presented via MHC class I and MHC class II respectively) present on T cells are required as co-receptors for TCR recognition of MHC-bound antigen.

1.3.1.2 T cell subsets

Figure 1-2 provides an overview of chicken T cell subsets as defined by their TCR, CD8 and CD4 expression. Mammalian T cells express one of two types of TCR; TCRαβ and TCRγδ. The TCRαβ type is present on $CD4^+$ and $CD8^+$ T cells and recognises antigen in the context of MHC molecules, a requirement to mounting adaptive immune responses. Chickens also have the TCRαβ however, in contrast to mammals, chickens have two types of β chain giving rise to two types of TCRαβ; $TCR\alpha\beta_1$ and $TCR\alpha\beta_2$ which differ in function and tissue distribution (Chen *et al.*, 1994). High proportions of $TCR\alpha\beta_1^+$ cells are observed in the intestinal epithelium and lamina propria, whereas $TCR\alpha\beta_2^+$ cells are largely absent from the intestine, however both are present in the spleen (Chen *et al.*, 1989). Upon depletion of

TCRαβ$_1$, birds exhibit reduced IgA in bile and in the lung mucosa and fail to produce IgA in response to mucosal immunisation however serum IgG and IgM are normal (Cihak *et al.*, 1991).

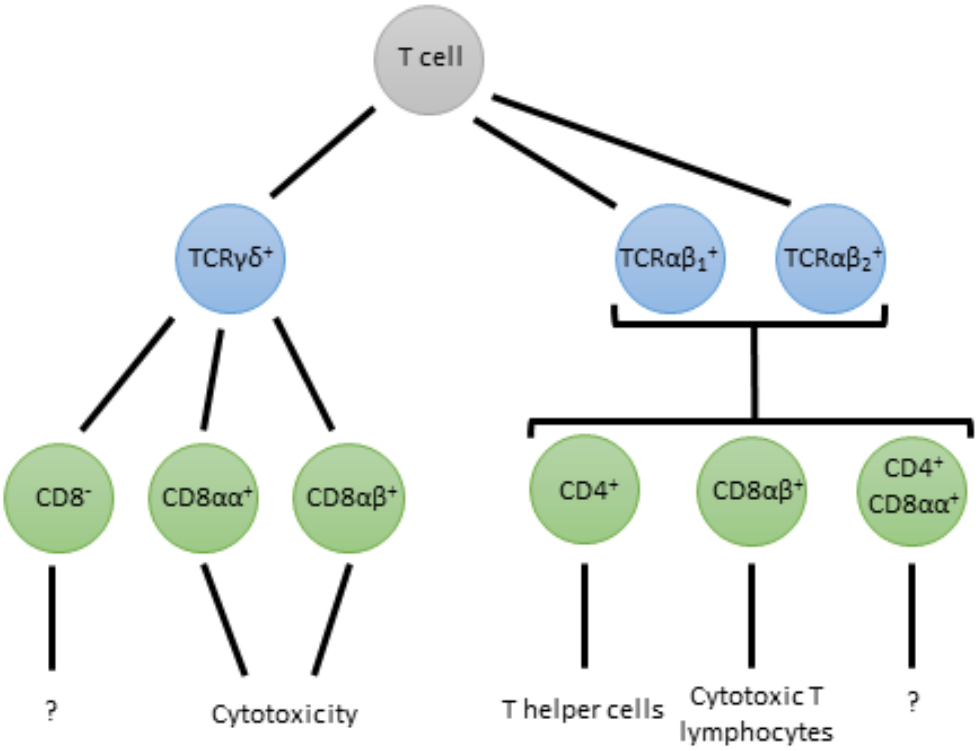

Figure 1-2: Chicken T cells as classified by TCR, CD4 and CD8 expression and their putative functions. Chicken T cells express one of three TCRs, and can be further subdivided by their expression of CD4, CD8αα homodimers and CD8αβ heterodimers. TCRγδ$^+$ T cells which co-express CD8αα or CD8αβ have higher levels of IFNγ, FasL and lymphotactin expression than their CD8$^-$ counterparts indicating an activated phenotype which may be involved in mediating cytotoxic activity and polarisation of a Th1 response through IFNγ production (Pieper *et al.*, 2008). TCRαβ$_1^+$ and TCRαβ$_2^+$ cells co-expressing the CD8αβ heterodimer are considered cytotoxic T lymphocytes (Merkle *et al.*, 1992) whereas those co-expressing CD4 are thought to be T helper cells, although their function has not been confirmed in the chicken. Additionally, chicken T cells include a TCRαβ$^+$CD4$^+$CD8αα$^+$ subset which proliferate in response to mitogens and can induce a graft versus host reaction, however their precise function is unknown (Luhtala *et al.*, 1997).

Cells expressing TCRγδ are much less common than those expressing TCRαβ in the lamina propria of the gut, however high numbers of TCRγδ$^+$ cells are present in the chicken gut epithelium (Bucy *et al.*, 1988). The chicken TCRγδ appears able to recognise antigens without the requirement of antigen processing and presentation, and can exhibit cytotoxic activity similar to that of CTLs (Chen *et al.*, 1994; Schild *et al.*, 1994). The frequency of T cells expressing TCRγδ in chicken peripheral blood leukocytes (PBLs) can reach up to 20%, much higher than that of humans and mice (Cooper *et al.*, 1991).

1.3.1.3 T helper cells

In mammals, depending on which cytokines are present, presentation of antigens via MHC class II to naïve CD4$^+$ T cells results in the activation of different signalling molecules, known as signal transducer and activator transcription factors (STATs), which upregulate the master transcription factors for different T helper (Th) subsets. These transcription factors drive the production of cytokines which polarise the naïve T cell towards a particular Th phenotype. Different Th subsets are defined by the cytokines that they produce, that drive their differentiation, and the transcription factors that they express (Table 1-2).

Table 1-2: Chicken and mammalian Th subsets. Highlighted red are features of Th responses not yet identified in the chicken. TGF-β4 is named TGF-β1 in mammals. CTL; cytotoxic T lymphocyte. Fox; forkhead box. GC; germinal centre. ROR; RAR-related orphan receptor. Tbet; T-box expressed in T cells. Treg; T regulatory cell. STAT; signal transducer and activator transcription factor.

Th subset	Driving cytokines	Signature cytokines	Signature transcription factors	Effector functions
Th1	IFNγ, IL-12	IFNγ	Tbet, STAT1	Phagocyte and CTL mediated defence against intracellular microbes
Th2	IL-4	IL-4, IL-5, IL-13	GATA3, STAT6	Stimulate antibody response to helminths
Tregs	IL-2, TGF-β4	TGF-β4, IL-10	FoxP3	Suppress immune responses, self tolerance
Th17	TGF-β4, IL-6, IL-1β	IL-17A, IL-17F, IL-21, IL-22, IL-23	RORγt, STAT3	Recruit neutrophils/heterophils, defence against extracellular bacteria and fungi
Th9	TGF-β4, IL-4	IL-9	PU.1, STAT6	Proinflammatory, involved in autoimmunity and allergy
Tfh	IL-21	IL-21	BCL6	GC formation, B cell selection and differentiation

The Th1/Th2 paradigm was first proposed by Mosmann and Coffman (1989), and describes the polarisation of adaptive responses towards a Th1 or Th2 phenotype. This paradigm appears to be conserved in the chicken; responses to intracellular pathogens such as Marek's disease virus (Volpini *et al.*, 1995; Xing *et al.*, 2000) and infectious bursal disease virus (Liu *et al.*, 2010) are governed by IFNγ/Th1 cells and responses to helminths such as *Histomonas meleagridis* are governed by IL-4/IL-13/Th2 cells (Powell *et al.*, 2009).

Polarisation of naïve T cells towards a Th1 phenotype is characterised by the production of IFNγ (Mosmann *et al.*, 1989). The IFNγ produced mediates the effector functions of the Th1 response including enhancing phagocytosis, release of ROS and RNS, cytokine production and antigen presentation in macrophages, enhancing NK and cytotoxic T lymphocyte (CTL) cytotoxicity and IFNγ production (Merlino *et al.*, 2002; He *et al.*, 2011) and, in mammals, is shown to enhance IL-12R expression on T cells to further polarise the Th1 response (Mullen *et al.*, 2001). In chickens, IL-12 is a heterodimer composed of IL-12α (p35) and IL-12β (p40) which is also shared with IL-23 (Degen *et al.*, 2004), however in mammals, IL-12α (p40) is shared with IL-23 (Aggarwall *et al.*, 2003). Polarisation of naïve T cells towards a Th2 phenotype is characterised by the production of IL-4 and IL-13 (Mosmann *et al.*, 1989). Th2 responses mediate immunity though B cell activation, proliferation and differentiation into plasma cells, antibody class-switching, antibody production and polarisation of macrophages towards a regulatory and wound-healing phenotype through their production of IL-10 and TGF-β. Chicken TGF- β4 is the equivalent of mammalian TGF-β1 (Halper *et al.*, 2004). In mammals, IL-5 is also a hallmark cytokine of Th2 responses and activates eosinophils and basophils (Kouro *et al.*, 2009). The absence of IL-5 during Th2 responses in the chicken may explain the lack of eosinophil and basophil activity observed. IL-5 is present in the chicken genome as a pseudogene (Avery *et al.*, 2004).

Th17 cells were first discovered in mammals by Harrington *et al.* (2005) and are characterised by their production of IL-17A, IL-17F, IL-21 and IL-22 (Korn *et al.*, 2009). Th17 cells exhibit microbicidal activity, are key regulators of inflammation and provide immunity at epithelial and mucosal barriers. All mammalian Th17-

driving and effector cytokines (IL-1β, IL-6, TGF-β4, members of the IL-17 family, IL-21, IL-22 and IL-23) have also been identified in the chicken. In mammals, the IL-17 family consists of six members; IL-17A-F. Five of these have been identified in the chicken genome; IL-17A-D and IL-17F (Kaiser *et al.*, 2005). IL-23 and its receptor, IL-23R (Welch, 2015), IL-21 (Rothwell *et al.*, 2012) and IL-22 (Kaiser *et al.*, 2005) have also been identified in the chicken genome. Only partial sequences of the Th17 transcription factor RORγt have been identified in the chicken genome, however, in mammals, RORγt is the master regulator of Th17 cells and its absence results in a loss of Th17 function (Ivanov *et al.*, 2006). In addition, $CD4^+$ splenocytes expressing intracellular IL-17A and IL-17F have been identified in the chicken (Walliser *et al.*, 2017). These factors suggest that chickens do possess Th17 responses as in mammals, which have been extensively characterised.

Th17 responses can be driven by either IL-6 and TGF-β or by IL-23 (Ghilardi *et al.*, 2007) and IL-23 is required for maintaining Th17 responses (Stritesky *et al.*, 2008). IL-17A and IL-17F are considered the main Th17 effector cytokines but are also a vital component of innate immunity at mucosal barriers and their defective production can lead to disease (Burkett *et al.*, 2015). Both these cytokines are pleiotropic in function and known functions of IL-17A and IL-17F in mammals include inducing production of proinflammatory cytokines by APCs, recruitment of neutrophils and macrophages to sites of inflammation, increasing production of anti-microbial peptides such as defensins and maintaining homeostasis at mucosal surfaces (Mills, 2008; Ishigame *et al.*, 2009; Song *et al.*, 2015). IL-17A and IL-17F drive the expression of IL-1β, IL-6 and in mammals, TNFα, to sustain inflammatory responses and drive IFNγ production in Th17 cells (Ivanov *et al.*, 2006). In addition,

IL-17A and IL-17F drive the production of anti-microbial peptides such as β-defensins and chemokines, by lymphocytes and epithelial cells at mucosal barriers (Kinugasa *et al.*, 2000).

Although IL-17A and IL-17F are primarily considered Th17 effector cytokines, they are also produced by innate cells. In mammals, NKT cells and TCRγδ$^+$ T cells are capable of producing IL-17A and IL-17F in mammals; TCRγδ$^+$ T cells when stimulated with IL-23 will produce IL-17A and IL-17F (Roark *et al.*, 2008; Martin *et al.*, 2009). Stimulated APC, NK and NKT cells are also capable of producing IL-22 (Goto *et al.*, 2009; Xu *et al.*, 2014; Fumgalli *et al.*, 2016). Early production of IL-17A and IL-17F has been observed in chickens following *Eimeria* infection (Kim, W. *et al.*, 2012) indicating that these cytokines are also involved in innate responses in chickens.

In mammals, IL-22 stimulates epithelial cells to produce chemokines (CXCL-8, CCL20) for the recruitment of neutrophils, and enhances their microbial responses through increased production of inducible nitric oxide synthase (iNOS) and mucins (Ziesché *et al.*, 2007; Raffatellu *et al.*, 2009). In addition, the induction of antimicrobial peptides from the RegIII family is dependent on IL-22 (Zheng *et al.*, 2008). IL-22, a member of the IL-10 family, is also important to tissue repair; IL-22-deficient mice exhibit delayed wound healing in the gut following dextran sodium sulphate (DSS)-induced colitis (Pickert *et al.*, 2009). IL-23 is not required for Th17 differentiation, however is required for sustaining Th17 responses (Ivanov *et al.*, 2007).

While important to immunity at mucosal barriers, inappropriate or uncontrolled Th17 responses are implicated in immunopathology and autoimmunity. IL-17A and IL-17F are responsible for chronic inflammation in rheumatoid arthritis (RA; Hot *et al.* (2011)), psoriasis (Adami *et al.*, 2014), Crohn's disease (Tesmer *et al.*, 2008) and ulcerative colitis (Zhang, H. *et al.*, 2016) and Th17 responses are considered a therapeutic target for resolution of disease (Hu *et al.*, 2011). Although IL-17A and IL-17F have been shown to drive IFNγ production, one study showed that normal levels of IFNγ are observed in the gut during Crohn's disease and ulcerative colitis (Rovedetti *et al.*, 2009). Another study showed that IL-17F was dispensable for the development of gut inflammatory disease but IL-17A was required (Ishigame *et al.*, 2009). In addition, IL-21 can drive inflammatory bowel diseases through induction of IL-17A; antibody-mediated depletion of IL-21 in mice results in an inability to develop colitis through a reduction in IL-17A production (Fina *et al.*, 2008). In addition, IL-21 can also drive gut inflammation through enhancing Th1 responses and it has also been suggested that IL-21 mediated Th1 responses are the cause of inflammation during Crohn's disease as higher numbers of $CD4^+$ T cells co-expressing IL-21 and IFNγ are observed in the lamina propria compared with numbers of $CD4^+$ T cells co-expressing IL-21 and IL-17A (Fina *et al.*, 2008). During inflammation in the gut in mice, IL-21 also enhances the formation of ulcers and the breakdown of tissue through inducing epithelial cells and fibroblasts to produce matrix metalloproteinases (MMPs), enzymes which degrade the extracellular matrix (Sengupta *et al.*, 2007).

In mammals, T regulatory cells (Tregs) are characterised by the expression of CD4 and CD25, their ability to produce IL-10 and their expression of the Forkhead box P3

(FoxP3) transcription factor. In the chicken, higher IL-10 and TGF-β4 is produced in $CD4^+CD25^+$ T cells than those that lack CD25 indicating that chickens may have Tregs (Shanmugasundaram *et al.*, 2011), but given that CD25 is considered an activation marker, further characterisation is required to determine if these are true Tregs. Additionally, FoxP3, the defining Treg transcription factor, has not yet been identified in the chicken genome although has been identified in birds such as the ground tit, peregrine falcon and saker falcon genomes (Denyer *et al.*, 2016).

Th9 cells are a Th cell subtype more recently discovered in mammals which express IL-9 and IL-10. Induction of Th9 cells requires both IL-4 and TGF-β and the transcription factor STAT6 (Goswami *et al.*, 2012). However, this subtype has not yet been identified in the chicken, though the relevant cytokines to induce Th9 and IL-9 itself have been identified in the chicken (this laboratory, unpublished). Follicular T helper (Tfh) cells are an additional T helper subtype found in mammals but which have not been formally identified in the chicken. Driven by and producers of IL-21, Tfh cells are involved in formation and maintenance of germinal centres (GCs) of lymphoid organs and mediating the selection and survival of B cells and their differentiation into antibody-producing plasma cells and memory B cells (Ma *et al.*, 2012).

1.1.2 Humoral Immunity

In the developing chicken embryo, haemopoeitic stem cells originating in the bone marrow relocate to the Bursa of Fabricius, where a diverse B cell receptor (BCR) and antibody repertoire is generated through somatic gene conversion (by Ig gene rearrangement in the bone marrow in mammals; reviewed by Hirano *et al.* (2011)). Naïve B cells then migrate to the periphery and upon recognition of the antigen by

the BCR, B cells express antigen via MHC class II molecules to $CD4^+$ Th cells, and alongside co-stimulation with CD40/CD40L become activated and undergo proliferation and maturation. Some of these B cells form GCs in secondary lymphoid tissues and, with help from Th cells and follicular DCs (fDCs), undergo affinity maturation and Ig class switching (reviewed in Avian Immunology, 2nd Edition, Chapter 4).

Chickens have three Ig heavy chain isotypes, IgM, IgA and IgY, compared with five in mammals, IgM, IgA, IgG, IgD and IgE. Chicken IgM is homologous in structure and function to that of mammalian IgM. IgM is produced after initial exposure to an antigen and, as in mammals, its production is usually transient (Davison *et al.*, 2008). Chicken IgY is the equivalent isotype to mammalian IgG. Similarly to mammalian IgG, chicken IgY is the predominant isotype found in chicken sera, with high concentrations found in serum yolk albumin and is produced shortly following IgM production during initial exposure to an antigen and in response to secondary exposure (Rose, 1974b; Davison *et al.*, 2008). Although considered similar to mammalian IgG, chicken IgY also has similarities to mammalian IgE in that it can mediate anaphylactic reactions (Carlander, 2002). Maternal IgY mediates passive immunity (Kowalczyk *et al.*, 1985). In both chickens and mammals, IgA is the predominant antibody isotype in bodily secretions (Bienenstock *et al.*, 1972; Lebacq-Verheyden *et al.*, 1972). No homologs of mammalian IgD or IgE have yet been identified in birds although some studies suggest that they do exist in birds (Carlander *et al.*, 1999).

1.4 Chicken gut mucosal immunity

1.4.1 Structure of the GALT

The inner wall of the gut is lined with projections known as villi, each of which is comprised of an outer epithelial layer of cells overlaying the lamina propria (Figure 1-1). Between each villi lie the crypt regions. The GALT is comprised of lymphoid cells in both the epithelium (intraepithelial lymphocytes; IEL) and lamina propria (lamina propria lymphocytes; LPL) and specialised lymphoid structures that line the gut and include the CT, PP and MD, the latter being unique to birds. The MD is the remnant of the yolk sac, which both provides a source of energy in the young chick and is the vehicle by which maternal IgY is passed from hen to chick. PP are interspersed throughout the gut and always at the ileocaecal junction (Befus *et al.*, 1980). In germ-free chickens, the development of GC in the CT and of lymphoid tissues is greatly reduced indicating that the microbiota of the gut is important to the development of lymphoid structures, and GC are also formed in response to antigen (Thorbecke *et al.*, 1957; Hedge *et al.*, 1999). Lymphoid structures comprise a specialised epithelium known as follicle-associated epithelium (FAE) overlying a lymphoid follicle. The FAE contains microfold (M) cells; large cells which exhibit microvilli on their upper surface and span the epithelial layer, resulting in sub-epithelial domains (Jeurissen *et al.*, 1999). In mammals, M cells are antigen-sampling cells which transport antigens through the epithelial layer towards the sub-epithelium, providing access to APC; the uptake of ferritin by chicken M cells has been demonstrated, however this was also observed in chicken epithelial cells (Jeurissen *et al.*, 1999). In addition to the FAE, M cells are also present in the Bursa (Bockman *et al.*, 1973). PP are lymphoid aggregates overlaid with villi which are wider and flattened in morphology in comparison with surrounding villi (Befus *et* al.,

1980). Directly underneath the FAE of PP are high numbers of lymphocytes and more organised lymphoid follicles which contain T cell-rich (with the majority expressing TCR$\alpha\beta_1$ and CD4) regions surrounding a B cell-rich GC, the site of mature B cell proliferation, differentiation, antibody class switching and the formation of memory B cells. B cells within this region express the chB6 antigen (Jeurissen *et al.*, 1989) and the majority of plasma cells present in the GC are IgY^+ with fewer IgA^+ and IgM^+ cells (Burns, 1982). Within the GC, fDCs, which express complement and Fc receptors, are also present (Jeurissen *et al.*, 1992) and are able to bind opsonised antibody for recognition of B cells via the BCR, allowing for selection of B cells into memory B cells. These fDCs also produce B cell-activating factor (BAFF), a B cell survival factor, and express CD40L, required as a costimulatory molecule for CD40 on B cells and therefore maintaining B cell proliferation and maintenance of the GC (Elgueta *et al.*, 2009). Naïve B cells in the periphery are also able to recognise and process antigens, which they can then present via MHC class II to Tfh cells at the T:B cell boundary of the GC. Th signalling, including cytokine and BAFF production, results in B cell proliferation and Ig secretion (Vainio *et al.*, 1984). In response to Th cell and fDC signals, B cells can differentiate into short-lived plasma cells which produce low-affinity antibodies or into memory B cells (Yasuda *et al.*, 1998). The CT are structurally and functionally similar to the PP and are located at the entrance to each caeca.

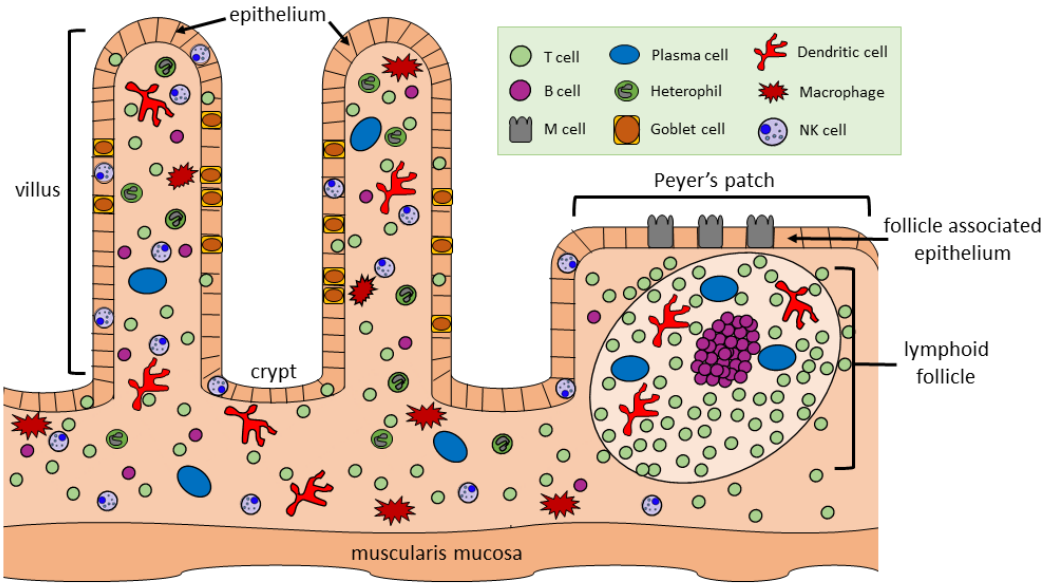

Figure 1-3: Schematic representation of immune cells in the small intestine of chickens and mammals. Adapted from Avian Immunology (2nd edition).

The presence of Paneth cells in the chicken has been suggested as the intestinal epithelium is a site of production of lysozyme and β-defensins (Wang *et al.*, 2016). Mucus-producing goblet cells are found interspersed throughout the gut epithelium of chickens, although these cells tend to be absent from the FAE. The mucus produced by these cells forms a barrier between the epithelium and the external environment and is comprised of mucins. Nine mucin genes have been described in the chicken however, MUC2, a gel-forming mucin, is the main mucin in the intestine of both chickens and mammals (Gum *et al.*, 1994; Jiang *et al.*, 2013). IgA is also an important component of mucus in both chickens and mammals (Zhang *et al.*, 2015). In addition, gut epithelial cells of both mammals and chickens constitute a physical barrier between the host and the external environment and express MHC class I molecules. Under conditions of inflammation, mammalian epithelial cells can upregulate their expression of MHC class II allowing for interaction between gut

epithelial cells and T cells (Thelemann *et al.*, 2014) however little is known about these interactions in the chicken.

1.4.2 Intestinal LPLs and IELs

Immune cell populations in the gut include macrophages, heterophils, DCs, NK cells, B cells and T cells and these can be further subdivided by their location, either to the lamina propria (LPLs) or epithelium (IELs). In the chicken, the IEL population consists of NK and T cells expressing either TCRγδ or TCRαβ_1. A large fraction of IELs express the CD8αα homodimer, whereas circulating $CD8^+$ cells express the CD8αβ heterodimer. Relatively few IELs express CD4, similarly as to mammals (Hoggenmueller *et al.*, 1993). Many chicken T cell IELs express TCRγδ (Cooper *et al.*, 1991). B cells are largely absent from the chicken intestinal epithelium, although a subset of epithelial NK cells expressing the B cell marker, chB6, are present (Vervelde *et al.*, 1993a). Macrophages and heterophils are more or less absent from the chicken intestinal epithelium (Hoggenmueller *et al.*, 1993), however infection can lead to translocation of heterophils across the epithelium (Kogut, 2002). The location of NK and T cells in the epithelial layer means that they are some of the first cells to establish contact with invading pathogens and are mediators of innate immunity. NK and T cells are able to recognise pathogens via PRRs on their surface, and in doing so, orchestrate inflammatory responses through the production of proinflammatory cytokines and cytotoxic activity (Chai *et al.*, 1988). $TCR\gamma\delta^+$ cells have been shown to be capable of cytotoxic activity, cytokine production, including IFNγ, and regulatory functions (Quere *et al.*, 1990; Chen *et al.*, 1994).

The lamina propria is populated with a more diverse array of cell types than the epithelium including T cells, B cells, macrophages, DCs, NK cells and heterophils.

In the chicken, the vast majority of LPLs are T cells, B cells, macrophages and DCs with some NK cells. In chickens, T cells in the lamina propria are mainly $CD4^+$, $CD8\alpha^+$, $TCR\alpha\beta_1^+$ cells although some $TCR\gamma\delta^+$ cells are present (Bucy *et al.*, 1988). Of the $TCR\alpha\beta_1^+$ population, the majority of cells co-express CD4 as opposed to CD8. Of the $TCR\gamma\delta^+$ LPL population, some are $CD8^+$ but the population of $CD4^+TCR\alpha\beta_1^+$ T cells is much larger and $TCR\alpha\beta_2^+$ cells are largely absent from the intestine (Char *et al.*, 1990). Adaptive immunity in the gut is characterised by increased numbers of $CD4^+$, $CD8^+$and $TCR\alpha\beta_1^+$ T cells and $chB6^+$ B cells in the lamina propria. NK cells in the lamina propria are mostly TCR^-chB6^-, but do possess an intracellular CD3 complex and surface CD8αα, are similar to mammalian NK cells in morphology and display cytotoxicity against the LSCC-RP9 tumour cell line, an NK cell target (Göbel *et al.*, 1994).

The B cells in the lamina propria are mainly IgA-secreting and account for the large concentrations of IgA observed in the intestinal mucosa in comparison with IgM and IgY (Lebacq-Verheyden *et al.*, 1972). Chicken IgA is transported through the epithelial layer through interaction with the polymeric Ig receptor (Rose *et al.*, 1981).

Macrophages (expressing the chicken mannose receptor (MRC)1L-B and colony stimulating factor (CSF)1R) also constitute a large population of cells in the lamina propria and many heterophils are also present (Mast *et al.*, 1998; Wells *et al.*, 1998). During infection, macrophages are able to recognise pathogens and mediate direct pathogen killing though phagocytosis and production of ROS and RNS, help mediate inflammatory responses through cytokine production, and act as a bridge between the innate and adaptive response, through antigen presentation and production of Th-

priming cytokines. Heterophils infiltrate the gut early on during infection and contribute to pathogen destruction and recruitment of other cells through expression of proinflammatory cytokines (Kogut, 2002). Heterophils and macrophages are also producers of anti-microbial peptides such as β-defensins and cathelicidins, contributing to innate mucosal immunity (Evans *et al.*, 1994; Zhao *et al.*, 2001).

1.5 Immune response to *Eimeria*

Infection of chickens with *Eimeria* induces both innate and adaptive responses, including increased heterophil activity, NK cell activity, T cell activation and antibody production and the outcome is dependent on a host of factors including the spp. of *Eimeria*, the genetics of the host and the severity of infection.

1.5.1 Innate responses to *Eimeria*

Eimeria infection results in an inflammatory immune response characterised by increased production of proinflammatory mediators (reviewed by Min *et al.* (2013)) and infiltration and activation of innate cells such as macrophages and heterophils, capable of phagocytosis and release of ROS and RNS to control the pathogen (Petrone *et al.*, 2002; Lillehoj *et al.*, 2004). It is likely that APC, including macrophages and DCs, fulfil multiple roles during *Eimeria* infection including phagocytosis, production of proinflammatory, regulatory and Th-promoting cytokines, production of ROS and RNS (such as H_2O_2 and NO) and antigen presentation for the development of cell-mediated and humoral responses. During first exposure to *E. tenella* sporozoites, significantly more sporozoites are located next to or within macrophages compared to that of immune chickens indicating that macrophages are important in the first line of defence against *Eimeria* (Vervelde *et al.*, 1996). Alternatively, it has been suggested that macrophages are involved in the

transport of sporozoites to the crypt regions as indicated in early studies where large numbers of *E. tenella* and *E. acervulina* sporozoites were observed within macrophage-like cells (Challey *et al.*, 1959; Doran, 1966). Increased macrophage numbers have also been identified in the gut following *E. acervulina* primary infection and *E. maxima* secondary challenge in chickens (Cornelissen *et al.*, 2009), *E. falciformis* infection in mice (Schmid *et al.*, 2014) and during *E. bovis* infection in cattle (Taubert *et al.*, 2009) and therefore it follows that macrophages are important cells in the response to *Eimeria*.

While little is known about the direct responses of macrophages to *Eimeria* infection *in vivo*, the response of various chicken macrophage cell lines to various *Eimeria* antigens has been examined. Chow *et al.* (2011) investigated the response of a cell line, characterised by Rath *et al.* (2003) as having macrophage-like properties, to various recombinant surface antigens (rSAGs) expressed on *E. tenella* sporozoites. The cell line, named HTC cells, increased nitrite production and *IL1B*, *IFNG*, *IL10* and *NOS2* messenger RNA (mRNA) production in response to *E. tenella* surface antigens (SAGs) 4 and 12 and *E. tenella* merozoite crude lysate. In the same cell line in response to *E. acervulina* sporozoites, increased *IL18* expression was also observed and differential Th1-promoting responses were observed in response to *E. tenella* (*IFNG* was upregulated), *E. maxima* and *E. acervulina* (*IFNG* was downregulated) sporozoites (Dalloul *et al.*, 2007). To date, the role of DCs in *Eimeria* infection has not been widely explored due to the lack of clearly defined markers to distinguish chicken DCs from macrophages, although some success in reducing oocyst shedding and increasing weight gains has been observed in using so

called DC-derived exosomes loaded with *E. tenella*, *E. maxima* or *E. acervulina* antigens to immunise chickens prior to challenge (delCacho *et al.*, 2012).

Heterophils form one of the first lines of defence against invading pathogens that display high levels of phagocytosis and can undergo processes such as oxidative burst and degranulation. Increased heterophils infiltrate the mucosa following *E. tenella* infection (Petrone *et al.*, 2002) and heterophils (as did macrophages) isolated from peripheral blood showed increased transcripts of *TLR4* and *TLR15* when stimulated with both live and heat-killed *E. tenella* oocysts (Zhou *et al.*, 2013). Formation of the neutrophil extracellular trap (NET) by bovine neutrophils has been observed *in vitro* in response to *E. bovis* sporozoites (Behrendt *et al.*, 2010) and in caprine neutrophils to *E. arlongi* sporozoites (Silva et al 2014). The formation of HETs in response to *Eimeria* by chicken heterophils has not yet been reported, but chicken heterophils do have the ability to form HETs similarly as in mammals (Chuammitri *et al.*, 2009).

NK cells, present in the intestinal epithelium, are one of the first innate cells to come into contact with *Eimeria* sporozoites therefore it is likely they are an important first line of defence against *Eimeria*. Hong et al. (2008) demonstrated that chicken NK lysin, an anti-microbial and anti-tumour peptide expressed by NK cells and T cells, demonstrates cytolytic activity when incubated with *E. acervulina* and *E. maxima* sporozoites *in vitro*. Following *E. acervulina* infection, NK lysin expression was upregulated from 7 dpi onwards, at 4 dpi with *E. maxima* infection and 3 dpi and then 6 dpi onwards with *E. tenella* infection in chicken IELs (Hong *et al.*, 2006a). Another study found that IEL NK cell activity decreased initially following *E. maxima* and *E. acervulina* infection in chickens but increased at 8 dpi and following

homologous challenge (Lillehoj, 1989). In contrast to these findings, *E. vermiformis* challenge experiments in mice revealed no significant role for NK cell activity during infection. Mice with defective NK cell cytotoxic activity were not more susceptible than control mice to *E. vermiformis* infection and similar levels of parasite replication were observed in both groups of mice (Rose *et al.*, 1995). Additionally, Smith *et al*., (1994) found that NK cell activity correlated with enhanced control of infection with *E. vermiformis*, however treatment of mice with an anti-aslio GM (anti-NK cell) antibody did not reduce control of the parasite. *Eimeria* antigens, including profilin, enhance human NK cell activity and IFNγ production and are being explored as potential adjuvants for treatment of infectious disease and cancer (Aylsworth *et al.*, 2013).

1.5.2 Cell-mediated immunity to *Eimeria*

Eimeria is highly immunogenic and elicits a high degree of protection to homologous challenge, but following heterologous challenge with different *Eimeria* spp. and strains within a spp., very little or no cross-protection is conferred (Rose *et al.*, 1962; Rose, 1967; Long, 1974; Smith *et al.*, 2002). T cells are an important aspect to primary and secondary *Eimeria* infection and in the development of protective immunity (Rose *et al.*, 1970). It appears that $CD4^+$ T cells coordinate and induce protective immunity and $CD8^+$ T cells execute effector functions to control parasite development during primary and secondary infection. Both $CD4^+$ and $CD8^+$ T cells increase following *Eimeria* infection. During *E. tenella* infection in chickens, the population of both $CD4^+$ and $CD8^+$ T lymphocytes increased the lamina propria in both naïve and immune chickens (Vervelde *et al.*, 1996). Following *E. maxima* infection, $CD4^+$ T cells infiltrated the lamina propria and $CD8^+$ cells increased in

both the lamina propria and the epithelium of the chicken jejunum (Rothwell *et al.*, 1995). Swinkels *et al.* (2006) also reported an increased percentage of $CD8\alpha^+$ cells in the duodenum of day old chicks following *E. acervulina* infection, but birds of this age failed to increase IFNγ production following infection. Increased numbers of $CD4^+$ T cells were observed in the jejunum, caecum and duodenum following single infection with *E. maxima*, *E. tenella* and *E. acervulina* respectively, however increased numbers of $CD8^+$ T cells were observed in the duodenum of *E. acervulina*–infected birds only. However, co-infecton with all three of these *Eimeria* spp. resulted in increased numbers of $CD8^+$ T cells in both the duodenum and caecum (Cornelissen *et al.*, 2009). Increased numbers of CTLs ($CD8\beta^+TCR\gamma\delta^-$) were also observed following primary *E. tenella* infection, but these increases were less pronounced following secondary and tertiary infection (Wattrang *et al.*, 2016b).

Various studies have also examined the effects of CD4 and CD8 depletion on immunity to *Eimeria*. Antibody-mediated depletion of CD4 during *E. tenella* infection enhanced oocyst shedding, but not during *E. acervulina* infection and that CD4-depletion did not affect the development of protective immunity (Trout *et al.*, 1996). The same study showed that in CD8- or TCRαβ-depleted chickens, reduced oocyst shedding occurs following *E. tenella* or *E. acervulina* primary infection but increased following secondary infection. During *E. acervulina* infection in chickens, intestinal $CD8^+$ T cells did not increase after primary infection, but increased with secondary infection, indicating a role in protective immunity (Trout *et al.*, 1996). These studies indicate that $CD4^+$ T cells are required for immunity during primary infection, whereas $CD8^+$ T cell effector functions are required for immunity to *Eimeria* following secondary exposure to the parasite.

Studies in rats and mice have indicated that $CD4^+$ T cells are important in immunity to primary infection and in the development of protective immunity. During *E. nieschultzi* infection, athymic rats develop no resistance to secondary infection and pass a greater number of oocysts during primary infection (Rose *et al.*, 1979). Rose *et al.* (1992) showed that pre-treatment of mice with an anti-CD4 antibody prior to *E. vermiformis* and *E. pragensis* infection resulted in increased faecal oocyst shedding and reduced immunity to subsequent homologous challenge, whereas anti-CD8α treated mice had increased faecal oocyst counts after secondary infection, indicating that $CD8\alpha^+$ T cells are important to controlling *Eimeria* during secondary challenge.

$TCR\gamma\delta^+$ cells are present in the intestinal epithelium and lamina propria although their importance during *Eimeria* infection is not well understood. In response to *E. acervulina* an increase in $TCR\gamma\delta^+$ cells is observed in the duodenum (Swinkels *et al.*, 2006) and in both a slow- and fast-growing chicken line increased TCRγδ+ cells were present in the duodenum 7 days after primary infection with *E. acervulina* and at 4 days after secondary challenge (Choi *et al.*, 2000; Swinkels *et al.*, 2007). In murine studies, TCRαβ-deficient mice had higher oocyst outputs than TCRαβ/γδ-deficient mice following *E. vermiformis* infection and adoptive transfer of $TCR\gamma\delta^+$ cells from the mesenteric lymph nodes (MLN) of both naïve and previously infected mice resulted in decreased oocyst shedding (Smith *et al.*, 2000a), indicating that $TCR\gamma\delta^+$ cells are important to protection against *Eimeria* but do not help impart specific protective immunity. Another study found that, in the absence of $TCR\gamma\delta^+$ cells, exacerbated immunopathology was observed during *E. vermiformis* infection (Roberts 1996), indicating that $TCR\gamma\delta^+$ cells may have a regulatory role during *Eimeria* infection. However in contrast, another study in mice challenged with *E.*

vermiformis, depletion of TCRγδ$^+$ IELs with a GL3 monoclonal antibody had no effect on faecal oocyst count in the mice or on immunity to secondary challenge (Rose *et al.*, 1996).

1.5.2.1 T helper responses to *Eimeria*

During *Eimeria* infection, increased IFNγ is produced from early time points onwards thus contributing to the innate inflammatory response and orchestrating the Th1 response. Increased IFNγ is produced at the site of infection in chickens infected with *E. acervulina*, *E. maxima* and *E. tenella* (Byrnes *et al.*, 1993; Rothwell *et al.*, 2000; Shirley *et al.*, 2007). Furthermore, pre-treatment of chickens with recombinant IFNγ resulted in improved weight gains compared to those not treated with IFNγ following infection with *E. acervulina* (Lowenthal *et al.*, 1997). In chickens also pre-treated with recombinant IFNγ, the ability of *E. tenella* to replicate within the host was greatly reduced and as a result, chickens given IFNγ reduced oocyst output following infection (Choi *et al.*, 1999). Breed *et al.* (1997) suggested that at 8 dpi with *E. tenella*, a subset of antigen-specific CD4$^+$ T cells circulate within the blood, primed to proliferate and produce IFNγ in response to secondary exposure of *E. tenella* sporozoites. A similar population appears to exist in the spleen which migrate towards sites of infection following secondary *E. tenella* challenge (Rothwell *et al.*, 2000). During *E. falciformis* and *E. pragensis* infection in mice, IFNγ deficient mice display severe pathology compared with those with intact IFNγ production and have reduced weight gain at both primary and secondary infection (Rose *et al.*, 1992; Pogonka *et al.*, 2010).

A Th2 immune response to *Eimeria* has not yet been observed. However, the Th2 response drives antibody-mediated immunity and a small number of roles for

humoral immunity have been observed in response to *Eimeria* infection (Davis *et al.*, 1979; Zigterman *et al.*, 1993), and these are described in section 1.5.3.

Immunity to other protozoan parasites including *Toxoplasma* (Dupont *et al.*, 2012), *Leishmania* (Castellano *et al.*, 2009), *Cryptosporidium* (Ehigiator *et al.*, 2007) and *Plasmodium* (Radosevic *et al.*, 2010) is also mediated by Th1 responses. During *T. gondii* infection, IFNγ is important to resistance and enhances phagocytosis by macrophages and cytotoxic activity by CTL (Ely *et al.*, 1999). In addition, IL-12 produced by APC induces IFNγ production by NK, $CD4^+$ and $CD8^+$ cells (Hunter *et al.*, 1995). However, Th2-related cytokines appear to be important for regulating Th1 responses and cell mediated immunity. IL-10 reduces macrophage IL-12 production, thereby reducing IFNγ production by NK and Th1 cells (D'Andrea *et al.*, 1992). In addition, IL-10 synergises with IL-4 and TGF-β to reduce the development of Th1 responses and therefore reducing subsequent immunopathology (Oswald *et al.*, 1992). Resistance to *L. major* is dependent on the polarisation of the Th response towards a Th1 phenotype; wild type mice are resistance compared to $IFN\gamma^{-/-}$ mice (Swihart *et al.*, 1995) where enhanced Th2 responses are observed (Wang *et al.*, 1994). During *Cryptosporidium parvum* infection, antibody-mediated depletion of IL-12 results in reduced IFNγ production and increased disease severity (Urban *et al.*, 1996). However, during *Cryptosporidium* infection in mice, increased IL-4-producing T cells are observed during the resolution of infection, indicating they are important in establishing humoral immunity and preventing prolonged infection (Aguire *et al.*, 1998).

Th17 cells have a role in promoting pro-inflammatory mediators and recruiting neutrophils to sites of infection in mammals. During infection with *E. tenella*, transcripts of *IL1B*, *IL6*, *IL17A* and *TGFB4* (the cytokines which promote Th17 differentiation) are up-regulated in the caecum of infected chickens and increasing numbers of heterophils infiltrating the caecum have been observed. *IL17A* and *IL17F* mRNA were upregulated in the jejunum of *E. maxima*-infected chickens (Kim *et al.*, 2012; Kim *et al.*, 2014) and *IL17F* increased in the caecum of *E. tenella*-infected chickens (Kim *et al.*, 2012). In IL-17 antibody-neutralised chickens, increased mRNA expression of Th1 cytokines, IL12 and IFNγ, was observed suggesting that Th17 cells down regulate Th1 responses (Zhang *et al.*, 2013). In murine *E. falciformis* infection, IFNγ$^{-/-}$ mice had increased immunopathology and morbidity due to an excessive Th17 (IL-17 and IL-22) response. However, a novel anti-parasitic role for IL-22 was observed, whereby IL-22-dependent antimicrobial peptides (RegIIIβ and RegIIIγ) were present in the intestinal tissue of infected mice (Stange *et al.*, 2012).

During immunity to protozoans other than *Eimeria*, it is unclear if Th17 responses contribute to increased immunopathology or help to clear infection. In mice, Th17 cells play a role in *Trypanosome cruzi* infection, regulating parasite-induced myocarditis by down-regulating IFNγ, TNF-α and IL-12 (Guedes *et al.*, 2010). In addition, antibody-neutralisation of IL-17A in mice infected with *T. cruzi* resulted in increased parasite burden (Mou *et al.*, 2010). However, in IL-17RA$^{-/-}$ mice challenged with *T. gondii*, mice display increased parasite burden in the gut, spleen and brain (Kelly *et al.*, 2005) and higher levels of IL-17A-producing CD4^{+} T cells

are observed in the spleen and liver of susceptible mice compared with resistant mice following *T. congolense* infection (Mou *et al.*, 2010).

1.5.3 Humoral response to *Eimeria*

Although immunity to *Eimeria* tends to be mainly cell-mediated, humoral immunity is also involved. High titres of *Eimeria*-specific IgY, IgA and IgM antibodies are detectable in serum and intestinal secretions from approximately 9 to 20 days post infection (Rose, 1974a; Trees *et al.*, 1989). The response of B cell deficient chickens to *Eimeria* has been studied through abrogation of the Bursa of Fabricius. Hormonally and chemically bursectomised chickens exhibit increased oocyst shedding and clinical disease in response to *E. maxima* and *E. acervulina* compared to non-bursectomised control chickens, although chickens still displayed substantial immunity during secondary infection, including decreased oocyst production, when compared with primary infection and control birds (Rose *et al.*, 1979). In another study, hormonal bursectomy of chickens had no significant effect on the immune response to *E. tenella* unless treated with cyclosporin A, when reduced resistance was observed (Lillehoj, 1987). These findings lead to the conclusion that immunity to *Eimeria* in chickens is predominantly T lymphocyte-dependent although a minor role exists for B cell antibody-mediated immunity.

Passive immunity also has a role in protection against *Eimeria*. Maternal antibodies are transferred to the chick hatchling via the yolk and infection with *E. maxima* in hens results in a high degree of passive immunity to the same species in chicks and partial protection against challenge with *E. tenella* (Smith *et al.*, 1994). Studies have also examined the effects of serum transfer from *Eimeria*-immune birds to *Eimeria* naïve birds. Antibodies in chicken sera after *Eimeria* infection provided passive

immunity to chickens challenged with the same species (Rose, 1974b). However, serum taken from *E. tenella*-immune fowls and transferred to *E. tenella*-naïve birds did not provide any protection (Pierce *et al.*, 1963).

The role of IgA during *Eimeria* infection has also been studied. Chickens immunised with *E. tenella* oocysts had increased *E. tenella* sporozoite-specific IgA titres in the bile at 9 days post-immunisation compared to control chickens. However, at 18 days post-immunisation, bile IgA titres decreased but were still higher than those of control birds (Rose *et al.*, 1987). Secretory IgA, present in the gut lumen of immune chickens, is thought to block *E. tenella* sporozoite entry into host cells (Davis *et al.*, 1979; Zigterman *et al.*, 1993).

1.6 Aims and Hypothesis

The overarching aim of this study was to identify biomarkers of resistance to *Eimeria* infection in the chicken. By identifying biomarkers of *Eimeria* resistance, it will be possible to test if they are suitable predictors of resistance in commercial broiler populations, providing a basis for selection of resistant chickens for breeding. In order to identify biomarkers of resistance, knowledge of the immune response to *Eimeria* is first required. This study therefore aimed to further phenotype the innate immune response to *Eimeria*, further characterise the role of Th17 responses during infection and identify biomarkers of *Eimeria* resistance and susceptibility in chickens which display natural resistance and susceptibility to infection.

As *Eimeria* elicits an inflammatory immune response and protective immunity in chickens, it is likely that *Eimeria* sporozoites are recognised by cells capable of driving inflammation and adaptive responses. The hypothesis is that chicken APC

recognise *Eimeria* sporozoites via TLRs present on the cell surface, respond to sporozoites by producing proinflammatory and Th-driving cytokines and in doing so, help to facilitate both the primary immune response to infection and protective immunity in response to primary infection or vaccination. To test this hypothesis, the responses of chicken bone marrow derived-macrophages and DC stimulated with an *E. tenella* oocyst crude lysate and recombinant immune mapped protein (IMP)1 and apical membrane antigen (AMA)1 from *E. maxima* and *E. tenella*, two potential vaccine candidates, will be investigated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to detect changes in the expression of proinflammatory and Th-promoting cytokines. A reporter gene assay will be used to determine if IMP1 and AMA1 are potential ligands for chicken TLRs.

Th17-associated cytokines are upregulated following infection with *Eimeria* spp. (Kim *et al.*, 2012; Kim *et al.*, 2014) and during *E. tenella* infection, neutralisation of IL-17A reduced immunopathology in chickens (Zhang *et al.*, 2013). The hypothesis is that Th17 responses contribute to immunopathology during *E. maxima* and *E. tenella* infection in a commercial broiler line. This hypothesis will be tested by orally infecting Ross 308 broilers with *E. maxima* or *E. tenella* and characterising the Th17 response following infection by using RT-qPCR to analyse the expression of Th17-associated cytokines in the gut following infection. Analysis of gut tissue by immunocytochemistry (ICC) will also highlight changes to cell subpopulations during infection.

White Leghorn chicken lines 15I and C.B12 exhibit inverse susceptibility and resistance to *E. maxima* and *E. tenella* (Bumstead *et al.*, 1995; Smith *et al.*, 2002).

The hypothesis is that since these lines vary in their resistance and susceptibility to *E. maxima* and *E. tenella*, their immune responses during infection will differ. To test this hypothesis, both these lines will be infected with *E. maxima* or *E. tenella* and differences in cytokine expression in the gut of the two lines determined by RT-qPCR. In addition, ICC will be used to characterise differences in cell subpopulations in the gut of the two lines following infection.

Chapter 2 Materials and Methods

2.1 Bacterial and molecular techniques

2.1.1 Bacterial cultures and storage

2.1.1.1 JM109 culture and preparation of glycerol stocks

Plasmids were grown in competent JM109 *Escherichia coli* (Promega). Single colonies were inoculated into 5 ml Lysogeny Broth (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 8.56 mM NaCl (pH 7.0)) and incubated overnight at 37°C, 5% CO_2 at 200 rpm. Cells were pelleted at 1,500 x *g* for 5 min and plasmids were extracted using the QIAprep Spin Miniprep or Maxiprep kit (Qiagen) as per the manufacturer's instructions, or glycerol stocks prepared by re-suspending cells in 15% glycerol (Fisher Scientific), 50% Super Optimal Broth (SOB; 20 g/L tryptone, 5 g/L yeast extract, 8.56 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$) and stored at -80°C.

2.1.2 Sub-cloning techniques

2.1.2.1 Primer design

Primers for PCR (Sigma) were designed using plasmid sequences as templates. Primers were approximately 20 bp in length, contained a guanine/cytosine content of 40 to 60% and had a melting temperature of approximately 58°C. For amplification of genes with the addition of both a 5' and 3' restriction site, primers were designed with additional restriction sites at both ends.

2.1.2.2 Amplification of target genes by PCR

Target genes were amplified using the Phusion High Fidelity PCR kit (New England Biosciences). Reactions were in 10, 25 or 50 µl volumes containing 50 to 100 ng template DNA, 0.75 mM $MgCl_2$, 0.1 µM each primer, 1 X Phusion HF reaction buffer, 1 U Phusion HF polymerase, 0.1 µM dNTPs (Promega) and nuclease-free

water (Ambion) to the final volume. PCR was performed using a Peltier Thermocycler (MJ Research) to the following program:

Denaturation step (one cycle):

98°C for 3 min

First annealing step (five cycles):

95°C for 1 min

X°C for 30 s (where X equals the melting temperature of the primers minus any additional restriction sites)

72°C for 1.5 min (30 s of elongation was allowed for every kb of target)

Second annealing step (thirty cycles):

95°C for 1 min

Y°C for 30 s (where Y equals the melting temperature of the primers including any additional restriction sites if any)

72°C for 1.5 min (30 s of elongation was allowed for every kb of target)

Elongation step (one cycle):

72°C for 10 min

In PCR where primers did not include additional restriction sites to be added to the target gene, amplification was performed by thirty-five cycles of one annealing step using the appropriate melting temperature for the primer pair. Reaction products were screened for the presence of the inserted gene using 1% agarose gel electrophoresis (2% agarose for short products).

2.1.2.3 TA Cloning into the pGEM®-T Easy Vector

A-tailing reactions were performed using the *Taq* DNA Polymerase kit (Invitrogen) in a 10 µl reaction containing 1 U *Taq* polymerase, 1 X PCR buffer, 2.5 mM $MgCl_2$, 0.5 µM dATP (Promega) and 4 µl PCR product. Reactions were for 30 min at 72°C in a Peltier Thermocycler. Varying quantities of A-tailed product were ligated into

pGem®-T Easy using the T4 DNA ligase kit (Promega) in 10 µl reactions containing 50 ng pGem®-T Easy vector, 1 U T4 ligase and 1 X Rapid Ligation Buffer. Ligation reactions were at RT for 1 h or 4°C overnight.

2.1.2.4 JM109 Transformation

JM109 cells were thawed on ice. Two µl plasmid DNA and 50 µl JM109 cells were mixed gently in an Eppendorf, incubated on ice for 20 min, at 42°C for 45 s then for 2 min on ice. The cells were resuspended in 950 µl SOB and incubated at 37°C, 5% CO_2 for 1 h at 200 rpm. Cells were centrifuged at 1,000 x *g* for 10 min to pellet, resuspended in 200 µl SOB and 100 µl spread onto duplicate LB agar (LB medium with 1.5% agar) plates and incubated overnight at 37°C, 5% CO_2.

Bacteria transformed with pGEM®-T Easy or pSecTag2 A-based plasmids were selected with 100 µg/ml ampicillin (Fisher Scientific). To screen bacteria transformed with pGEM®-T Easy-based plasmids by blue/white screening, 100 µl 100 mg/ml isopropyl β-D-1-thiogalactopryanoside (IPTG; Fisher Scientific) and 20 µl 50 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Fisher Scientific) was spread onto LB agar plates and air-dried prior to use. To confirm the presence of insert genes in transformed colonies, PCR was performed as in section 2.1.2.2. A pipette tip was used to pick up colonies which were placed into 50 µl nuclease-free water, incubated at 95°C for 5 min and 2 µl used as a template for the PCR.

2.1.2.5 Enzymatic digestion of plasmid DNA

Restriction digest reactions were prepared in nuclease-free water containing 1 µg plasmid DNA, 1 U restriction enzyme (Promega, New England Biosciences or Roche) and 1 X reaction buffer (provided with restriction enzyme) and incubated at

37°C for 1 h. When two restriction enzymes incompatible with the same reaction buffers were required, digestions were performed consecutively. Product was purified using the QIAquick PCR Purification kit (Qiagen) according to the manufacturer's instructions before the second digestion. Digested DNA was purified by 1% agarose gel electrophoresis and the QIAquick gel extraction kit (Qiagen) used according to the manufacturer's instructions to purify bands of interest prior to ligation using the T4 DNA ligase kit (section 2.1.2.3). Plasmid concentrations were determined using the NanoDrop ND-1000 spectrophotometer.

2.1.2.6 Plasmid sequencing

Plasmids were sequenced using the Sanger method by Edinburgh Genomics (https://genomics.ed.ac.uk). Sequencing reactions contained 1 µl 3.2 pmol/µl primer (Sigma) and 5 µl plasmid DNA (containing 200 to 500 ng plasmid). Sequences were analysed using the Lasergene 10 SeqMan Pro software (DNASTAR®).

2.1.3 Expression and analysis of recombinant proteins in mammalian cell lines

2.1.3.1 Diethylaminoethyl (DEAE)-Dextran Approach

COS-7 cells were seeded at 6 x 10^6 cells in a T75 cell culture flask (section 2.3.1.1) and incubated overnight at 37°C, 5% CO_2 to 75-90% confluence. Fifty-two µg plasmid DNA was added to 2.5 ml Dulbecco's Modified Eagle Medium (DMEM; Sigma) and 15 µl 100 mg/ml DEAE-dextran (Sigma), mixed and dispersed evenly over the cells. After 30 min incubation at 37°C, 5% CO_2, 7.5 ml DMEM, 10% FBS (Gibco) and 75 mM chloroquine was added and the cells were incubated for 2.5 h at 37°C, 5% CO_2. The cell monolayer was washed once in PBS without dislodging the cells. The PBS was removed and the cells were shocked with 10% DMSO (Sigma) for 2 min, washed with PBS and 15 ml DMEM, 10% FBS, 2 mM L-glutamine

(Gibco), 1% non-essential amino acids (NEAA; Gibco) was added. The cells were incubated overnight at 37°C, 5% CO_2. The medium was then replaced with serum-free medium (DMEM, 2 mM L-glutamine and 1% NEAA). Supernatants were harvested four days later by removing medium from cells and centrifuging at 500 x *g* for 5 min to remove cell debris. The serum-free DMEM was replaced and a second harvest was performed two days later.

2.1.3.2 Polyethylenimine (PEI) Approach

HEK293T cells were seeded at 3 x 10^6 cells in a T75 cell culture flask and incubated at 37°C, 5% CO_2 overnight to 70% confluence. Seven µg plasmid DNA, 500 µl DMEM and 35 µl PEI (Sigma) were mixed, incubated at RT for 25 min then added to the cell medium and incubated for 4 h at 37°C, 5% CO_2. Medium was replaced with 15 ml of serum-free medium (DMEM, 2 mM L-glutamine). Supernatants were harvested four days post transfection by removing medium from cells and centrifuging at 500 x *g* for 5 min to pellet cell debris. The serum-free DMEM was replaced and a second harvest performed two days later.

2.1.3.3 Dot blot

Incubation and washing steps were on a plate shaker at RT. A vacuum blotter was used to draw 100 µl of cell supernatant onto a nitrocellulose membrane (Optitran). The membrane was washed in PBS, 0.5% Tween 20 (PBST) for 30 min, blocked in PBS, 0.5% casein (Sigma) for 1 h then washed twice for 10 min in PBST. Membranes were incubated in anti-penta-His antibody (Qiagen) diluted 1:2,000 in PBS, 0.5% casein for 1 hr then washed three times in PBST for 10 min. Membranes were incubated for 1 h with goat anti-mouse IgG-HRP (Southern Biotech) diluted 1:1,000 in PBS, 0.5% casein then washed three times in PBST for 10 min. To detect

proteins, the Pierce™ ECL Western Blotting Substrate kit (Thermo Scientific) was used as per the Manufacturer's instructions and visualised using High Performance Chemiluminescence Film (GE Healthcare) developed using a Medical Film Processor (Konica). Recombinant chicken IL-10 His-tagged protein (supplied by Dr Zhiguang Wu, The Roslin Institute) was used as a positive control and untransfected COS-7 and HEK293T supernatant as negative controls.

2.1.3.4 LI-COR® Western Blot for protein quantification

Mini-PROTEAN® TGX™ precast gels (Biorad) were used with the Mini-PROTEAN® Tetra Cell electrophoresis system (Biorad) for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transfer steps. To perform SDS-PAGE, 5 µl sample or protein standard was prepared in 10 µl reactions with 1 X NuPAGE LDS sample buffer (Invitrogen), 500 mM DTT (Serva) and nuclease-free water to the final volume, incubated at 98°C for 5 min then placed on ice. The protein standard used was a His-tagged Ovine Prion Protein (OvPrP-His; supplied by Dr Andy Gill, the Roslin Institute). Precision Plus Protein™ Dual Colour Standard (BioRad) was used as a ladder. Ten µl sample, ladder or standard was loaded to each well and electrophoresis run at 120 V for 1 h. Separated samples were transferred onto nitrocellulose membranes using 25 mM tris-HCl, 192 mM glycine, 20% methanol) for 1.5 h at 40 V on ice.

Membranes were blocked in Odyssey® Blocking Buffer (LI-COR® Biosciences) at RT for 1 h then rinsed in PBS and incubated with anti-penta-His antibody (Qiagen) at a 1:2,000 dilution in Odyssey® Blocking Buffer overnight at 4°C. Membranes were washed four times for 5 min in PBST (0.1%) then incubated at RT for 1 h with IRDye 680 goat anti-mouse antibody (LI-COR® Biosciences) diluted 1:10,000 in

PBST (0.1%), 25% Odyssey® Blocking Buffer. From addition of the IRDye 680 goat anti-mouse antibody onwards all washing and incubation steps were in the dark. Membranes were washed four times for 5 min in PBST (0.1%), twice for 5 min in PBS then imaged using the LI-COR Odyssey® Imaging Scanner (LI-COR® Biosciences). Images were analysed using Image Studio Lite version 5.0. A standard curve was prepared using the intensity of bands produced by the OvPrP-His standard protein which was used to calculate the concentration of protein in the samples.

2.2 Quantification of gene expression

2.2.1 RNA extraction

To isolate RNA from tissues and cells the RNeasy® Mini Kit (Qiagen) was used according the manufacturer's instructions.

To extract RNA from cultured BMM and BMDC, media was completely removed from wells and cells washed with 3 ml PBS. The PBS was removed and 600 µl Buffer RLT, 1% β-mercaptoethanol (Sigma) was added. Plates were wrapped in parafilm and stored at 4°C for 24 h to 2 weeks before the RNA extraction protocol was followed.

To extract RNA from tissues, tissues were collected into RNAlater (Ambion) and stored at 4˚C for 24 h then at -20°C until RNA extraction was performed. To extract RNA, 20 to 30 mg tissue was weighed and placed into a 2 ml Eppendorf safe-lock micro test tube (Scientific Laboratory Supplies) containing a 5 mm steel bead (Qiagen) and 600 µl Buffer RLT, 1% β-mercaptoethanol. Tissues were homogenised

using the TissueLyser II (Qiagen) for 2 min at 20 Hz. Homogenised tissues were centrifuged at 13,000 rpm for 3 min and the RNA extracted from the supernatant.

2.2.2 TaqMan® Real Time-Quantitative PCR

Primers (Sigma) and probes (Eurogentec) were designed using Primer Express software (Applied Biosystems) and are in Table 2-1. Primers were 8-40 bases long with a guanine/cytosine content of 20-80%, a melting temperature of 58-60°C (and less than 2°C difference between a pair) and the last 5 nucleotides containing less than two guanine or cytosine residues. Probes were 9-40 bases long with a guanine/cytosine content of 20-80%, a melting temperature 10°C higher than that of the primer pair and not starting with a guanine residue at the 5' end. Both primers and probes were designed so they did not contain more than four consecutive identical bases. For each target gene, at least one of the primers or the probe spanned an intron/exon boundary.

Table 2-1: Primers and probes for TaqMan® RT-qPCR. Optimum primer concentrations were determined by lab members by titrating primer concentrations (1 to 0.1 μM) with a positive RNA standard. Positive RNA standards were produced by lab members by transfecting COS-7 cells plasmids containing the gene of interest or were RNA extracted from LPS stimulated-HD11 cells.

Target Gene	Primers and Probe Sequences	Standard RNA	Primer Conc. (μM)
28S	Probe 5' (FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA) 3' Forward 5'-GGCGAAGCCAGAGGAAACT-3' Reverse 5'-GACGACCGATTTGCACGTC-3'	HD11 stimulated with LPS	0.6
IL1B	Probe 5' (FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA)3' Forward 5'-GCTCTACATGTCGTGTGTGATGAG-3' Reverse 5'-TGTCGATGTCCCGCATGA-3'	ExCOS-7 IL-1β mRNA	0.4
IL2	Probe 5' (FAM)-ACTGAGACCCAGGAGTGCACCCAGC-(TAMRA) 3' Forward 5'-TTGGAAAATATCAAGAACAAGATTCATC-3' Reverse 5'-TCCCAGGTAACACTGCAGAGTTT-3'	ExCOS-7 IL-2 mRNA	0.6
IL4	Probe 5' (FAM)-AGCAGCACCTCCCTCAAGGCACC-(TAMRA) 3' Forward 5'-AACATGCGTCAGCTCCTGAAT-3' Reverse 5'-TCTGCTAGGAACTTCTCCATTGAA-3'	ExCOS-7 IL-4 mRNA	0.4
IL6	Probe 5' (FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA) 3' Forward 5'-GCTCGCCGGCTTCGA-3' Reverse 5'-GGTAGGTCTGAAAGGCGAACAG-3'	ExCOS-7 IL-6 mRNA	0.2
IL10 (1)	Probe 5' (FAM)-CGACGATTCGGCGCTGTCACC-(TAMRA) 3' Forward 5'-CATGCTGCTGGGCCTGAA-3' Reverse 5'-CGTCTCCTTGATCTGCTTGATG-3'	ExCOS-7 IL-10 mRNA	0.4
IL10 (2)	Probe 5' (FAM)-CCAACTGCTCAGCTCTGAACTGCTGGAT-(TAMRA) 3' Forward 5'-GAAATTAAGGACTATTTTCAATCCAGAGA-3' Reverse 5'-ACAGACTGGCAGCCAAAGGT-3'	ExCOS-7 IL-10 mRNA	0.8
IL12A	Probe 5' (FAM)-CCAGCGTCCTCTGCTTCTGCACCTT-(TAMRA) 3' Forward 5'-TGGCCGCTGCAAACG-3' Reverse 5'-ACCTCTTCAAGGGTGCACTCA-3'	ExCOS-7 IL-12α mRNA	0.4
IL15	Probe 5' (FAM)-AAGTTGCAAATCTTGCATTTCCATTTTTCCA-(TAMRA) 3' Forward 5'-TAGGAAGCATGATGTACGGAACAT-3' Reverse 5'-TTTTTGCTGTTGTGGAATTCAACT-3'	ExCOS-7 IL-15 mRNA	0.8
IL17A	Probe 5' (FAM)-ACAACCGCTTCCCCCGCTTGG-(TAMRA) 3' Forward 5'-CATGGGATTACAGGATCGATGA-3' Reverse 5'-GCGGCACTGGGCATCA-3'	ExCOS-7 IL-17A mRNA	0.4
IL17F	Probe 5' (FAM)-CAGGAATCGGTCTCTCGCTCCTTGG-(TAMRA) 3' Forward 5'-TGACCCTGCCTCTAGGATGATC-3' Reverse 5'-GGGTCCTCATCGAGCCTGTA-3'	ExCOS-7 IL-17F mRNA	0.6
IL18	Probe 5' (FAM)-CCGCGCCTTCAGCACGGATG-(TAMRA) 3' Forward 5'-AGGTGAAATCTGGCAGTGGAAT-3' Reverse 5'-ACCTGGACGCTGAATGCAA-3'	ExCOS-7 IL-18 mRNA	0.8
IL21	Probe 5' (FAM)-TGCTGCATACACCAGAAAACCCTGGG-(TAMRA) 3' Forward 5'-AAAAGATGTGGTGAAAGATAAGGATGT-3' Reverse 5'-GCTGTGAGCAGGCATCCA-3'	ExCOS-7 IL-21 mRNA	0.2
IL23R	Probe 5' (FAM)-AGGACAGCCACACCTACATCCAAGAGGAC-(TAMRA) 3' Forward 5'-ACAGCCATGAGGAAGAGTTCTTTT-3' Reverse 5'-CATACACCGACACGTTGATGTG-3'	ExCOS-7 IL-21R mRNA	0.4
TGFB4	Probe 5' (FAM)-ACCCAAAGGTTATATGGCCAACTTCTGCAT-(TAMRA) 3' Forward 5'-AGGATCTGCAGTGGAAGTGGAT-3' Reverse 5'-CCCCGGGTTGTGTTGGT-3'	ExCOS-7 TGF-β4 mRNA	0.1
NOS2	Probe 5' (FAM)-TCCACAGACATACAGATGCCCTTCCTCTTT-(TAMRA) 3' Forward 5'-TTGGAAACCAAAGTGTGTAATATCTTG-3' Reverse 5'-CCCTGGCCATGCGTACAT-3'	LPS stimulated HD11 mRNA	0.2
IFNG	Probe 5' (FAM)-TGGCCAAGCTCCCGATGAACGA-(TAMRA) 3' Forward 5'-GTGAAGAAGGTGAAAGATATCATGGA-3' Reverse 5'-GCTTTGCGCTGGATTCTCA-3'	ExCOS-7 IFN-γ mRNA	0.6

The amplification and detection of specific mRNA were achieved using the AB 7500 FAST Real-Time PCR System (Applied Biosystems). Reactions were in 10 µl volumes containing 2.5 µl RNA (total RNA diluted 1:5 in nuclease-free water for all genes except 28S where the dilution was 1:500), 5 µl 2 X FAST Master Mix (Applied Biosystems), 0.125 µl RNase Inhibitor (20 U/µl; Applied Biosystems); 0.125 µl Multiscribe Reverse Transcriptase (50 U/µl; Applied Biosystems), 0.5 µl diluted primers, 0.25 µl probe (5 µM) and 1.5 µl nuclease-free water. The following PCR program was used; 48°C for 30 min, 95°C for 20 s, 40 cycles of 95°C for 3 s, 60°C for 30 s.

Data were collected during the cycling stage. Standard curves were generated from the C*t* values of the serially diluted positive RNA standard to obtain the slope of the line with the following equation:

$$y = mx + c$$

By obtaining the slope of the standard curve generated by the serially diluted positive RNA standards the efficiency (E) of the reaction was calculated using the following equation:

$$E = 10^{-1/slope}$$

Corrected C*t* values were calculated by the following equation:

$$Normalised\ Ct = Ct + (N't - C't) \times \left(\frac{S}{S'}\right)$$

Where C*t* is the original cycle threshold obtained from the RNA sample, N'*t* is the mean of the 28S C*t* values across all samples within a plate, C'*t* is the mean 28S C*t*

values of triplicate wells for one sample, S is the slope generated by the standard curve for the gene of interest by serially diluting the positive standard RNA for that gene and S' is the slope generated by the standard curve of the 28S gene by serially diluting the positive RNA standard for 28S. Corrected 40-C*t* values were calculated by subtracting the normalised C*t* value from 40. Within an experiment, the threshold of detection for each gene was set to be within the linear phase of exponential amplification of the standards and was kept constant for genes that were measured across multiple plates.

2.3 Cell line culture

Techniques were performed in a Category 2 biosafety cabinet.

2.3.1 Maintenance of cell lines

2.3.1.1 Maintenance of COS-7 cells

COS-7 cells were maintained in DMEM, 10% FBS, 2 mM L-glutamine, 1 U/ml penicillin, 1 µg/ml streptomycin, 1% sodium pyruvate and 1% NEAA (complete COS-7 medium) at 37°C, 5% CO_2 and passaged at 70-80% confluence. Cells were washed as a monolayer with PBS and incubated for 5 min at 37°C, CO_2 with 5 ml Versene (Invitrogen), 10% trypsin (Invitrogen). Ten ml DMEM, 10% FBS was added to the flask and dissociated cells were transferred to a Falcon tube and washed twice by centrifuging at 500 x *g* for 5 min and re-suspending in 10 ml COS-7 medium. Cells were counted and reseeded at 7.5 x 10^6 cells per T75 cell culture flask.

2.3.1.2 Maintenance of HEK293T cells

HEK293T cells were maintained in DMEM, 10% FBS, 2 mM L-glutamine, 1 U/ml penicillin, 1 µg/ml streptomycin, 1% sodium pyruvate and 1% NEAA at 37°C, 5%

CO_2 in a T75 cell culture flask and passaged at 70% confluence. Cells were washed twice as a monolayer with PBS then incubated for 5 min at 37°C, 5% CO_2 with 5 ml Accutase (Gibco). Dissociated cells were collected into a Falcon tube and washed twice by centrifuging at 500 x *g* for 5 min and re-suspending in 10 ml HEK293T medium. Cells were counted and reseeded at 5.75 x 10^5 cells per T75 cell culture flask.

2.3.1.3 Maintenance of HEK293T-SEAP cells

HEK293T-SEAP cells were maintained in DMEM, 2 mM L-glutamine, 1 U/ml penicillin and 1 µg/ml streptomycin (complete HEK293T-SEAP medium) at 37°C, 5% CO_2 in a T75 cell culture flask and passaged at 70% confluence. Cells were washed twice as a monolayer with PBS and incubated for 1-2 min at 37°C, 5% CO_2 with 5 ml PBS, 20% TrypLE (Gibco). Dissociated cells were transferred to a Falcon tube and washed twice by centrifugation at 500 x *g* for 5 min and re-suspended in 10 ml HEK293T-SEAP medium. Cells were counted and reseeded at 5.75 x 10^5 cells per T75 cell culture flask.

2.3.2 SEAP reporter assay

Chicken TLR sequences were cloned into the pcDNA3 plasmid (Thermo Fisher Scientific) by Dr Sungwon Kim (The Roslin Institute). Twenty-four h prior to transfection, HEK293T-SEAP cells were passaged (section 2.3.1.3) and seeded at 4 x 10^4 cells/well in a flat bottomed 96-well tissue culture plate. Lipofectamine® 2000 (Thermo Fisher Scientific) was prepared by adding 0.4 µl Lipofectamine® 2000 to 25 µl DMEM per well. Two hundred ng plasmid was added to 25 µl DMEM per well. For transfections involving multiple plasmids, equal quantities of each plasmid were added to a total of 200 ng per well. Lipofectamine and plasmid solutions were

mixed then incubated at RT for 10 min. Medium was replaced with 100 µl DMEM and 50 µl Lipofectamine/plasmid added. Plates were incubated for 4 h at 37°C, 5% CO_2 after which 100 µl DMEM, 20% FBS was added. Cells were cultured overnight at 37°C, 5% CO_2, medium replaced with 100 µl HEK293T-SEAP medium and cells cultured for 24 h. Medium was replaced with ligands prepared in DMEM, 2% FBS and cells incubated for 18 h at 37°C, 5% CO_2. Twenty µl supernatant from each well was added to 100 µl Quanti-Blue solution (Invivogen) prepared as per the manufacturer's instructions. Colour change was developed for 45 min at 37°C before the optical density (OD) was measured at 650 nm in a Multi Ascent spectrophotometer (Thermo Electron Corporation). Negative controls were cells transfected with pcDNA3-YFP plasmid containing no gene, untransfected cells, cells stimulated with medium only and HEK293T or COS-7 mock-transfected cell supernatants. Positive control ligands are in Table 2-2.

Table 2-2: TLR ligands used as positive controls for the HEK293T-SEAP reporter gene assay.

TLR	Ligand	Description	Manufacturer
1LB/2A and 1LB/2B	Pam3CSK4	Synthetic triacylated lipopeptide, mimics the acylated amino terminus of LPS.	Invivogen
21	CpG-ODN	Synthetic DNA molecule of sequence 5' – gtc gtt gtc gtt gtc gtt – 3' containing cysteine nucleotides linked to guanine nucleotides by one phosphate group.	Eurofins genomics

2.4 Primary cell culture

Techniques were performed in a Category 2 biosafety cabinet.

2.4.1 Ethics statement

Chickens used for primary cells were culled by cervical dislocation and confirmation of death confirmed by decapitation in accordance with Schedule One methods as set out by the UK Home Office and in accordance with the Animals (Scientific Procedures) Act 1986.

2.4.2 J and Novogen Brown chicken lines

The J line is an outbred brown leghorn that originates from the Edinburgh Poultry Research Centre (Edinburgh). The Novogen Brown line is an outbred commercial layer line. Both lines were maintained at the National Avian Research Facility (NARF) based at the Roslin Institute. Birds were vaccinated against MDV, coccidiosis, NDV, IBV, IBDV, chicken anaemia virus, avian encephalomyelitis virus and infectious laryngotacheitis virus.

2.4.3 Culture of BMM and BMDC

Three to five-week-old J and Novogen Brown line chickens were culled and femurs and tibias aseptically removed into cold PBS. The bone marrow was flushed out with 10 ml PBS using a 21G needle, passed through a 70 µM strainer and cells washed by centrifugation at 400 x *g* for 5 min then re-suspended in 10 ml PBS. The cell suspension was underlaid with 10 ml Histopaque 1.077 (Sigma) and centrifuged for 20 min at 400 x *g* at RT (acceleration and brakes off). Cells were collected from the interface, made up to 20 ml with PBS, centrifuged at 400 x *g* for 5 min then re-suspended in 10 ml BMM medium (Rosewell Park Memorial Institute (RPMI)-1640 (Invitrogen), 10% FBS, 2 mM L-glutamine, 1 U/ml penicillin and 1 µg/ml

streptomycin) or BMDC medium (RPMI-1640, 10% chicken serum (Biosera), 2 mM L-glutamine, 1 U/ml penicillin and 1 µg/ml streptomycin). Cells were counted and adjusted to 1 x 10^6 cells/ml. For BMDC culture, recombinant chIL-4 and chCSF2 were added at a 1:200 dilution. For BMM culture, recombinant chCSF1 was added at a 1:200 dilution. Cells were seeded at 6 x 10^6 cells/well of a 6-well tissue culture plate and incubated at 41°C, 5% CO_2. On days 2 and 4 of culture, 2 ml medium was replaced with 2 ml fresh medium. Recombinant chCSF2, chIL-4 and chCSF1 were produced by previous lab members from COS-7 cells transfected with pCIneo plasmids (Promega) containing either chCSF1, chCSF2 or chIL-4 (Wu *et al.*, 2010; Garcia-Morales *et al.*, 2014).

To stimulate BMM and BMDC, stimulants were diluted in cytokine-free BMM or BMDC medium. Medium was removed from each well and replaced with 3 ml stimulant.

2.4.4 Preparation of *E. tenella* oocyst crude lysate

Sporulated *E. tenella* oocysts (Wisconsin strain; Doran *et al.* (1974)) were provided by Prof. Damer Blake (The Royal Veterinary College, UK). Oocysts (1 x 10^8) were washed twice by centrifugation at 1,700 x *g* for 5 min and re-suspending in 8 ml PBS. Approximately 8 ml 600 µm glass beads (Sigma) were added and oocysts were vortexed three times for 2 min. Oocysts were checked microscopically for adequate disruption of the oocyst wall and the presence of high numbers of sporocysts. Sporocysts were sonicated twice for 30 s then freeze/thawed three times on dry ice. The concentration of the lysate was determined using the Pierce 660 nM Protein Assay kit (Thermo Fisher Scientific) as per the manufacturer's instructions. The lysate was tested for LPS contamination using the Pierce LAL Chromogenic

Endotoxin Quantitation Kit (Thermo Scientific) according to the manufacturer's instruction.

2.4.4.1 Analysis of *E. tenella* oocyst crude lysate toxicity by FACS

BMM cultures were stimulated overnight with 1, 10 and 50 µg/ml crude lysate, 200 ng/ml LPS or medium only. Medium was replaced with BMM medium, 15 mM EDTA. Cells were harvested into a Falcon tube by pipetting, centrifuged at 400 x *g* for 10 min, resuspended in 10 ml BMM medium and counted by Haemocytometer. Cells were centrifuged at 400 x *g* for 10 min and resuspended in FACs buffer (PBS, 0.5% BSA and 0.05% NaN_3) at 1 x 10^7 cells/ml. One hundred µl cells was dispensed to wells of a 96-well U-bottomed plate. Plates were centrifuged at 300 x *g* for 3 min and the supernatant discarded. Cells were resuspended on a plate shaker and 50 µl FACS buffer alone or containing mouse anti-chicken CD45 antibody-FITC (clone LT40, Southern Biotech) at a 1:200 dilution was added. Plates were covered and incubated for 30 min on ice. Cells were washed three times as described and resuspended in 300 µl FACS buffer alone or containing SYTOX® Blue (Thermo Fisher Scientific) at a 1:1,000 dilution and incubated for 5 min on ice. Analysis was performed using a BD Fortessa (BD Biosciences) and cell populations analysed using FlowJo V10 (Tree Star).

2.5 *In vivo* experiments

2.5.1 Ethics statement

Animal experiments were performed under the authority of the UK Home Office project licence held by Prof. Fiona Tomley (PPL No. 70/7781; The Royal Veterinary College, UK) within the terms and conditions of the UK Home Office Animals (Scientific Procedures) Act 1986.

2.5.2 Bird lines

2.5.2.1 Ross 308 broilers

Ross 308 broilers were supplied by Aviagen. Birds were unvaccinated.

2.5.2.2 15I and C.B12 White Leghorns

Line 15I is a white leghorn line originating from the Avian Disease and Oncology Laboratory (ADOL; East Langsing, MI, USA). Line C.B12 is a white leghorn line originating from the University of Cambridge. Both flocks were maintained at the NARF (The Roslin Institute). Birds were unvaccinated.

2.5.3 Challenge models

2.5.3.1 Commercial bird trial

Ross 308 broilers (n=91) were divided into three groups; an *E. tenella*-infected (35 birds), an *E. maxima*-infected (35 birds) and an uninfected control group (21 birds). Birds were housed in separate rooms on littered pens. At three weeks of age, broilers were orally inoculated with 2 x 10^3 oocysts (in 0.5 ml water) of *E. maxima* (Wisconsin strain; Norton *et al*. (1976)) or *E. tenella* (Houghton strain; Joyner *et al*. (1969)) or left uninfected. Both *Eimeria* strains were provided by Prof. Damer Blake (The Royal Veterinary College, UK) and were harvested from infected chickens no more than one month prior to use. At each sampling time point there were five *E. tenella*-infected, five *E. maxima*-infected and three uninfected control birds. Sampling time points were 2, 3, 4, 5, 8, 11 and 15 dpi. The weights of all birds were recorded at 2 dpi and then again in individual birds prior to culling. Birds were sedated by intramuscular injection of 0.2 ml xylazine (2% Rompun) combined with 0.2 ml ketamine then culled by intraperitoneal injection of 200 mg/kg of body weight of pentobarbital. Cervical dislocation was performed as confirmation of death. At each time point, samples were taken from the jejunum, 1 cm anterior to the MD, and

the mid-caecum. Tissues were harvested from the left caecum for RNA extraction (section 2.2.1) and the right caecum for histology. For histology samples, 1 drop OCT medium (Thermo Fisher Scientific) was placed on a square of filter paper. Tissues were placed onto the OCT medium, snap-frozen on liquid nitrogen and stored at -80°C.

For quantification of parasite burden in the birds, the residual tissue of the two caeca were pooled within each bird of the *E. tenella*-infected group and 10 cm lengths of jejunum (spanning 5 cm either side of the MD) were collected from the *E. maxima*-infected group for total genomic parasite DNA.

2.5.3.2 Inbred line trial

Inbred chicken lines 15I (n=103) and C.B12 (n=96) were separated into six groups; two uninfected control groups (n=21 for each line), two *E. maxima*-infected groups (n=41 for 15I and n=39 for C.B12) and two *E. tenella*-infected groups (n=41 for 15I and n=36 for C.B12). The six groups were housed in one room in separate littered pens. At three weeks of age, birds were orally dosed with *Eimeria*. Birds in the *E. maxima* groups were inoculated with 100 *E. maxima* (Wisconsin; Norton *et al.* (1976)) oocysts in 1 ml water and birds in the *E. tenella* groups were inoculated with 200 *E. tenella* (Houghton; Joyner *et al.* (1969)) oocysts in 1 ml water. Both *Eimeria* strains were provided by Prof. Damer Blake (The Royal Veterinary College, UK). *E. maxima* oocysts were harvested within one month prior to use. *E. tenella* oocysts were harvested 6 months prior to use, and so a higher dose of 200 oocysts was chosen to account for any reduction in viability. Birds in the control groups were inoculated with 1 ml sterile water. The numbers of birds culled and sampled from on each time point are shown in Table 2-3..

Table 2-3: Number of birds culled at each time point during the trial.

Day Post Infection	**2**	**4**	**5**	**6**	**7**	**8**	**13**
Control group							
15I	3	3	3	3	3	3	3
C.B12	3	3	3	3	3	3	3
***E. maxima* group**							
15I	5	7	6	6	6	6	5
C.B12	5	6	6	6	6	5	5
***E. tenella* group**							
15I	5	6	6	6	6	6	6
C.B12	5	5	5	5	5	6	5

Bird weights were recorded two days prior to inoculation and then again prior to culling. Birds were culled by cervical dislocation and death confirmed by decapitation. Immediately following decapitation, blood was collected into a Falcon tube and stored at RT for at least 1 h, centrifuged at 1,500 x *g* and the serum stored at -20°C. Tissue samples were collected from the jejunum and caeca of control birds, the jejunum of *E. maxima*-infected birds and the caeca of *E. tenella*-infected birds. Jejunum was sampled 2 cm posterior to the MD. Caecum was sampled from the mid-section of each caecum. Samples were collected from the left caecum for RNA (section 2.2.1) and the right for histology by filling a plastic cryomould (15 x 15 x 5 mm; Fisher Scientific) with OCT medium into which tissues were placed, orientated and snap-frozen on a foil boat on liquid nitrogen. Cryomoulds containing frozen tissues were wrapped in foil and stored in zip-lock bags at -80°C.

For quantification of parasite burden in the birds, the residual tissue of the two caeca were pooled within each bird of the *E. tenella*-infected group and 10 cm lengths of jejunum (spanning 5 cm either side of the MD) were collected from the *E. maxima*-

infected group for genomic DNA extraction. For control birds, the jejunum and caecum tissues were pooled.

2.5.3.3 Quantification of parasite burden in the gut of infected birds by qPCR

Parasite genomic DNA was quantified by a qPCR targeting the RAPD-SCAR marker Tn-E03-1161 for *E. tenella* (Nolan *et al.*, 2015) and the MIC1 gene for *E. maxima* (Blake *et al.*, 2006). Targets were amplified by qPCR and Ct values used to calculate the starting quantities of parasite and host genome copy numbers using a standard dilution series of known copy numbers. The starting quantity of parasite genome was normalised against the *actB* gene of the host genome to obtain the total copy number of parasite genome per mg of tissue. All parasite quantification by qPCR was performed by Dr Matt Nolan (The Royal Veterinary College, UK).

2.5.4 Quantification of cytokines in serum by ELISA

To quantify IL-10 levels in the serum the capture antibody (ROS-AV164), biotinylated detecting antibody (ROS-AV163) and recombinant chIL-10-Fc were kindly provided by Dr Zhiguang Wu (The Roslin Institute; Wu *et al.* (2016)). All incubation steps were at RT, in a humidified container on a rocking platform (70 rpm) unless stated otherwise. All wash steps were performed by removing liquid from the plates and adding 200 µl PBST to each well. At no point were wells allowed to dry. Twenty-four h prior to running the assay, the capture antibody was diluted to 3 µg/ml in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3) and 50 µl dispensed to wells of a flat-bottomed 96-well plate and stored at 4°C. Wells were washed three times, blocked with PBS, 0.5% casein for 1 h then washed once before 50 µl samples and protein standards were added in duplicate. Plates

were incubated for 1 h, washed three times and 50 µl detecting antibody (1 µg/ml in PBS, 0.5% casein) was added to each well and incubated for 1 h. Plates were washed three times and 50 µl High Sensitivity Streptavidin-HRP (Pierce) was added at a 1:10,000 dilution in PBS, 0.5% casein. Plates were incubated for 1 h, then washed three times. One-Step Ultra TMB-ELISA Substrate Solution (Thermo Scientific) was equilibrated to RT and 50 µl dispensed to wells. Plates were incubated for 5 min at RT and 50 µl stop solution (2N H_2SO_4) was added. OD was measured at 450 nm using the Multiscan Ascent spectrophotometer. Serum IFNγ levels were quantified by Dr Sungwon Kim (Roslin Institute, UK) using the Chicken IFNγ CytoSet Kit (Invitrogen) according to the manufacturer's instructions. To calculate the concentration of IL-10 and IFNγ in serum, 4-parameter logistic regression was performed using the following formula:

$$y = d + \frac{(a-d)}{[1+\left(\frac{x}{c}\right)^{b}]}$$

Where *y* is the concentration of protein in the sample, *x* is the OD value, *a* is the minimum OD value that can be obtained (when the sample is negative), *d* is the maximum OD value that can be obtained, *c* is the point of inflection (the point of the curve midway between *a* and *d*), and *b* is the Hill's slope of the curve (the slope of the curve at *c*).

2.5.5 Quantification of IL-10 in tissue

Tissue was snap frozen at collection and stored at -80°C until use. Samples were prepared at 4°C. Twenty-five mg tissue was homogenised in 600 µl protein lysis buffer (20 mM Tris (pH 7.5), 100 mM NaCl, 0.5% NP-40 (IGEPAL® CA-630;

Sigma), 0.5 M EDTA, 0.5 mM phenylmethylsulphonyl fluoride (PMSF; Sigma), 0.5% protease inhibitor cocktail (Sigma)) using a TissueLyser II (Qiagen) at 25 Hz for 2 min twice, with incubation on ice in between homogenisation steps. Samples were centrifuged at 13,000 x *g* for 10 min at 4°C. The concentration of protein in the supernatant was determined using the BCA protein assay kit (Thermo Scientific) according to the manufacturer's instructions. The concentration of IL-10 was then determined by ELISA as in section 2.5.4 and the concentration of IL-10/mg of tissue calculated. Analysis of IL-10 in gut tissue was performed by Dr Sungwon Kim (The Roslin Institute, UK).

2.6 Immunocytochemistry (ICC)

2.6.1 ICC procedure

A Leica CM1900 cryostat was used to cut 7 µm sections which were picked onto Superfrost® glass slides (Thermo Scientific), air-dried overnight, fixed in acetone (0.75% H_2O_2) for 10 min at RT and air-dried for 5 min. Depending on section size, 50-100 µl diluted antibody was prepared per section. Primary antibodies (Table 2-4) were diluted in PBS and added to the slides ensuring sections were covered. A humidified box was used for all incubation steps which were conducted at RT for 1 h and washes were three times for 2 min with PBS in a Coplin jar. The Vectastain Elite ABC (Mouse IgG) Kit (Vector Laboratories) was used as follows. Two drops normal horse serum and 1 drop biotinylated antibody from the kit was added to 10 ml PBS and vortexed to mix. Slides were washed, excess PBS removed with lint-free tissue and sections incubated with the biotinylated antibody. To prepare the ABC reagent, 1 drop of reagent A (Avidin DH solution) and 1 drop of reagent B (biotinylated HRP) was added to 5 ml PBS, vortexed to mix and incubated for 20

min at RT. Slides were washed in PBS and incubated with the ABC reagent. Slides were washed and 3-Amino-9-ethylcarbazole (AEC) was used as a substrate for colour development of sections using the AEC staining kit (Sigma) as per the manufacturer's instructions. Slides were incubated with the AEC substrate for 4 min at RT, washed once in PBS and once in Milli-Q water. Slides were incubated for 2 min in haematoxylin Z (Cell Path), washed with tap water then incubated for 2 min in Scott's Tap Water (tap water, 2 % magnesium sulphate, 0.35% sodium bicarbonate). Slides were mounted in Aquamount AQ (Vector).

Slides were imaged using a Nikon Eclipse Ni microscope using ZEN lite 2012 software. Images were taken with 20 ms exposure, gamma correction at 0.75 and white balance set to a blank area of the slide. For images, the histogram thresholds were set to the maximum and minimum using the automatic feature in Zen lite 2012.

Table 2-4: Antibodies used in ICC staining.

Antibody (Clone Name)	**Specificity**	**Isotype**	**Source (Catalogue Number)**	**Dilution Used**
Mouse anti-chicken CD4 (CT-4)	Chicken CD4	IgG_1	Southern Biotech (8210-01)	1:400
Mouse anti-chicken CD8α (3-298)	Chicken CD8α	IgG_{2b}	Southern Biotech (8405-01)	1:400
Mouse anti-chicken TCRγδ (TCR1)	Chicken TCRγδ	IgG_1	Southern Biotech (8230-01)	1:400
Mouse anti-chicken TCRαβ/vβ$_1$ (TCR2)	Chicken TCRαβ$_1$	IgG_1	Southern Biotech (8240-01)	1:400
Mouse anti-chicken TCRαβ/vβ$_2$ (TCR3)	Chicken TCRαβ$_2$	IgG_1	Southern Biotech (8250-01)	1:400
Mouse anti-chicken monocyte/macrophage (KUL01)	Chicken mannose receptor 1 (MRC1) on monocytes, macrophages, interdigitating cells and microglia	IgG_1	Southern Biotech (8420-01)	1:800
Mouse anti-chicken Bu-1/ChB6 (AV20)	Chicken chB6, present on B cells and epithelial NK cells	IgG_1	Southern Biotech (8395-01)	1:800
Mouse anti-chicken CD25 (AV142)	AV142	IgG_1	Bio-Rad (MCA59259A)	1:400
Mouse anti-chicken Tim4S (JH9)	Chicken Tim4 short and long isoforms, present on APCs	IgG_1	Produced in house (Hu *et al.*, 2016)	1:400
Mouse anti-chicken CSF1R (ROS-AV170)	Chicken CSF1R	IgG_1	Produced in house (Garcia-Morales *et al.*, 2014)	1:100

2.6.2 Descriptive Quantification of Cell Populations in ICC Sections

Three units of measurement were adopted to quantify the different areas of the gut. For jejunum, two different units of measurement were used to quantify the cell populations present in the villi and the crypts. One unit of villus area was defined as a 300 µm length of villus midway along the length of the villus (Figure 2-1A). One unit of crypt area was defined as an area of 22,500 μm^2 over the crypts (Figure 2-1B). The crypt area was defined as the area between the bases of the villi and the muscle wall of the intestine. One section was scored per bird and for each section, three units of measurement over the villi and three units of measurement over the crypts were counted over three separate areas of each tissue section.

For the caecum, one unit of measurement was used. A caecal unit was defined as a 22,500 μm^2 area which was used to measure the population of cells in the caecum (Figure 2-1C). Over three separate areas of the tissue, at least three caecal units were counted in total.

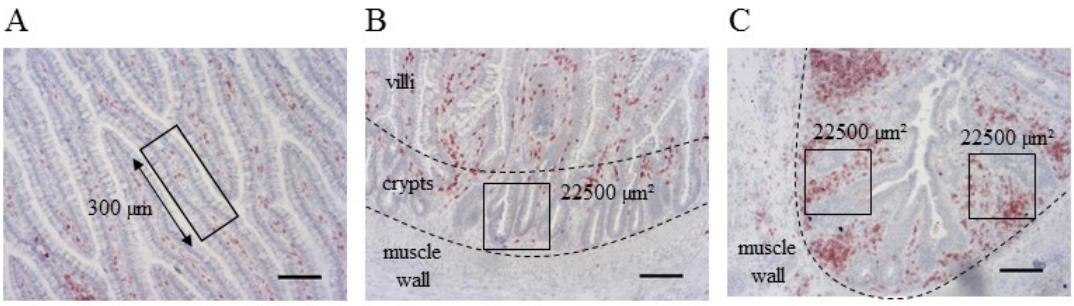

Figure 2-1: Units of measurement for counting cell populations in the intestine. For the jejunum villi, a 300 µm length of villi mid-way between the tips and the base of the villi was counted (A). For the jejunum crypts, an area of 22500 μm^2 was selected at random over the crypt areas (B). For caecal villi and crypts, an area of 22500 μm^2 was selected at random over both crypt and villi areas (C). Bars represent 100 µm.

For each antibody, control tissues were examined from 3, 5, 8 and 15 dpi and a baseline level of cells (Table 2-5) in the lamina propria was determined using the units of measurement defined above. The number of stained cells in the lamina propria of each unit of measurement was counted individually, compared to the baseline control levels of cells and scored according to the criteria as in Table 2-6. On the occasion where counts in controls exceeded the baseline level as in Table 2-5, the section was scored accordingly as in Table 2-6. Entire sections were also screened for clusters defined as 10 or more cells in contact with one another. If clusters were observed during scoring an automatic score of 3 was assigned. The number of cells present in the epithelium of each unit was counted and a score assigned as in Table 2-7. Sections were scored blind by an impartial third party to eliminate bias.

Table 2-5: Baseline levels of cells present in the lamina propria of uninfected control jejunum and mid-caecum tissues of Ross 308 broilers and lines 15I and CB12. All control tissues were analysed across 3, 5, 8 and 15 dpi and the number of stained cells were counted individually within appropriate units as described in this section. Per count, the number of cells did not exceed the numbers as described.

Cell Marker	**Jejunum**	**Mid-caeca**
CD4	≤ 20 per villus unit and ≤ 20 per crypt unit	≤ 20 per caecal unit
CD8α	≤ 10 per villus unit and ≤10 per crypt unit	≤ 10 per caecal unit
TCRγδ	≤ 10 per villus unit and ≤ 10 per crypt unit	≤ 5 per caecal unit
TCRαβ$_1$	≤ 10 per villus unit and ≤ 10 per crypt unit	≤ 20 per caecal unit
TCRαβ$_2$	Not observed	Not observed
CD25	≤ 20 per villus unit and ≤ 10 per crypt unit	≤ 20 per caecal unit
ChB6	≤ 5 per villus unit and ≤ 5 per crypt unit	≤ 5 per caecal unit
MRC1	≤ 30 per villus unit and ≤ 25 per crypt unit	≤ 30 per caecal unit
Tim4	≤ 30 per villus unit and ≤ 20 per crypt unit	≤ 30 per caecal unit

Table 2-6: Lamina propria scoring criteria.

Score	Criteria
0	No cells present in the lamina propria.
1	Baseline control level for each cell marker (as defined in Table 2-5). Cells scattered throughout the lamina propria.
2	An increase in the number of cells present in the lamina propria compared to the control baseline level (as defined in Table 2-5).
3	An increase in the number of cells present in the lamina propria compared to the control baseline level (as defined in Table 2-5) and clusters of cells present in the lamina propria either in the crypt regions and/or villi.
4	The majority of the lamina propria is stained due to the presence of high numbers of cells.

Table 2-7: Epithelium scoring criteria.

Score	Criteria
0	No cells present in the epithelium.
1	Very few cells present in the epithelium (<5 per jejunum villi, jejunum crypt or caecal unit).
2	Cells present in the epithelium (5-20 cells per jejunum villi, jejunum crypt or caecal unit).
3	High numbers of cells present in the epithelium (>20 per jejunum villi, jejunum crypt or caecal unit).

2.7 Statistical Analysis

All data were checked for normality and tested for statistical significance in Minitab 17. The statistical tests performed are detailed throughout this thesis.

Chapter 3 Innate responses of antigen presenting cells to *Eimeria* antigens *in vitro*

3.1 Introduction

Eimeria infection elicits an immune response that culminates in clearance of the parasite and development of protective immunity against reinfection with homologous species. In order to initiate an immune response to a pathogen, the pathogen must first be recognised by PAMPs via host PRRs. Currently, it is not known which *Eimeria* PAMPs are recognised by which chicken PRRs. A description of chicken PRRs is given in Chapter 1. TLRs are one of the main families of PRRs in both chickens and mammals and are likely involved in recognition of *Eimeria*. APCs including macrophages and DCs are vital to initiating an immune response. APCs have important roles in both innate and adaptive immunity and these roles include recognition and phagocytosis of pathogens, release of cytokines (proinflammatory, regulatory and Th-driving) and presentation of pathogenic molecules to T cells for induction of a pathogen-specific, adaptive immune response. The responses of macrophage cell lines to *Eimeria* antigens have been studied (Dimier *et al.*, 1998; Chow *et al.*, 2011), however current literature surrounding the response of chicken-derived macrophages and DCs is lacking. The hypothesis is that TLRs on the surface of epithelial cells and APC, recognise *Eimeria* and guide the innate inflammatory and adaptive Th1 immune response through the production of cytokines. The aim of this chapter was to study the responses of BMM and BMDC to the *Eimeria* parasite and recombinant *Eimeria* proteins and to determine which chicken TLRs recognise these *Eimeria* antigens.

In this study, BMM and BMDC responses to, and TLR recognition of, two *Eimeria* proteins were investigated. Immune mapped protein 1 (IMP1) is conserved across apicomplexan parasites (Cui *et al.*, 2012a; Cui *et al.*, 2012b) and is present on the

surface of *Eimeria* sporozoites (Jenkins *et al.*, 2015), is highly immunogenic, confers protection against *E. maxima* (Blake *et al.*, 2011) and is a potential vaccine candidate for *E. tenella* (Yin *et al*., 2013). A *T. gondii* IMP1 DNA vaccine also enhanced Th1 responses following challenge in mice and increased the length of survival time (Cui *et al.*, 2012b). Apical membrane antigen 1 (AMA1) is also conserved across apicomplexan parasites and interacts with rhoptry neck proteins (RONs) during host cell invasion. AMA1 is most highly expressed at the sporozoite stage during the *E. tenella* life cycle and use of an anti-EtAMA1 antibody reduced invasion of DF-1 cells by *E. tenella* sporozoites (Jiang *et al.*, 2012). An AMA1 DNA vaccine was protective and reduced oocyst output following *E. brunetti* infection in chickens compared with chickens not given the vaccine (Hoan *et al.*, 2014).

Bone marrow cells can be cultured *ex vivo* and driven towards a macrophage (BMM) phenotype with recombinant CSF-1 (Garceau *et al.*, 2010) or towards a DC (BMDC) phenotype with recombinant chicken IL-4 and CSF-2 (Wu *et al.*, 2010). To characterise the responses of APC to the *Eimeria* parasite and potential *Eimeria* vaccine candidates, chicken BMM and BMDC were stimulated with EtAg from *E. tenella* and recombinant IMP1 and AMA1 from both *E. maxima* and *E. tenella*. Changes in the expression of genes associated with the inflammatory (IL-1β, IL-6 and iNOS), Th1 (IFN-γ and IL-12α), Th2 (TGF-β4 and IL-4) and regulatory (IL-10) response were measured by RT-qPCR.

Since IMP1 and AMA1 are both immunogenic, it is expected that they are both recognised by chicken TLRs. Here, a SEAP reporter gene assay was utilised to determine if either IMP1 (EtIMP1 and EmIMP1 from *E. tenella* and *E. maxima*

respectively), AMA1 (EtAMA1 and EmAMA1 from *E. tenella* and *E. maxima* respectively) or EtAg were recognised by chicken TLR1LB/2A or TLR1LB/2B heterodimers or TLR21. TLR1LA, TLR1LB, TLR2A and TLR2B are the functional homologs of the mammalian TLR1/6/10 family. Heterodimers formed between chicken TLR1LA or B and TLR2A or B recognise lipoproteins and peptidoglycans (Higuchi *et al.*, 2008) as do heterodimers formed between TLR2 and TLR1, 6 or 10 in mammals (Nishiguchi *et al.*, 2001; Nakao *et al.*, 2005). TLR1LA/2B heterodimers in chickens and TLR1/2, TLR2/10 and TLR2/6 heterodimers in mammals recognise diacylated and triacylated lipoproteins. Peptidoglycan is recognised by TLR1LB/2A heterodimers in the chicken and TLR2 heterodimers in mammals. Chicken TLR21 is the functional homologue to mammalian TLR9 and both recognise CpG oligodeoxynucleotides (ODN; reviewed by Brownlie *et al.* (2009)).

3.2 Results

3.2.1 Response of BMM and BMDC to *E. tenella* oocyst crude lysate

To examine the role of APC in inducing an inflammatory response during *E. tenella* infection, BMM and BMDC were stimulated with EtAg as in section 2.4. RNA was extracted from cells and RT-qPCR analysis performed for proinflammatory genes including *IL1B* (Figure 3-1), *IL6* (Figure 3-2) and *NOS2* (Figure 3-3) as described in section 2.2. EtAg stimulation significantly increased the expression of *IL1B*, *IL6* and *NOS2* mRNA in BMM.

EtAg stimulation increased the expression of *IL1B*, *IL6* and *NOS2* mRNA in BMDC. However, due to a pipetting error, for BMDC at 3 hours post stimulation (hps), results from one bird for medium-only and LPS-stimulated treatments are missing.

As a result, the statistical power of the test was reduced, affecting the statistical outcome and results were not significant at this time point.

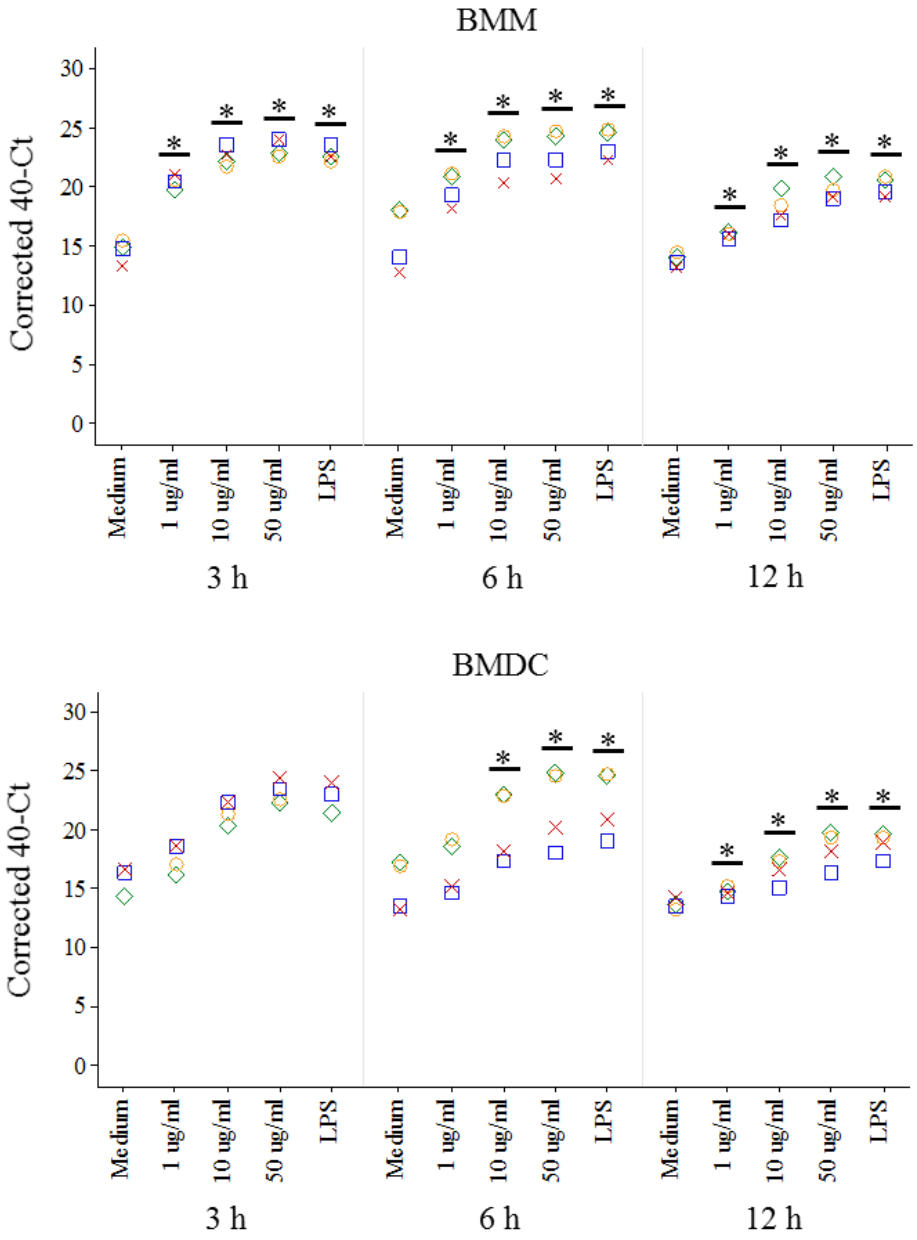

Figure 3-1: *IL1B* mRNA expression in BMM and BMDC following stimulation with EtAg. BMM and BMDC were cultured until day 6 then incubated with medium alone (negative control), 1, 10 or 50 µg/ml of EtAg or 200 ng/ml of LPS (positive control) for 3, 6 and 12 hours. RNA was extracted and the expression of *IL1B* mRNA measured by RT-qPCR. Data are presented as corrected 40-Ct values for four individual birds except for BMDC at 3 h where data are shown for three birds for medium and LPS-treated cultures. Individual colours denote data from individual birds. Asterisks indicate significant differences compared to medium controls at $p \leq 0.05$ according to the Mann-Whitney U test.

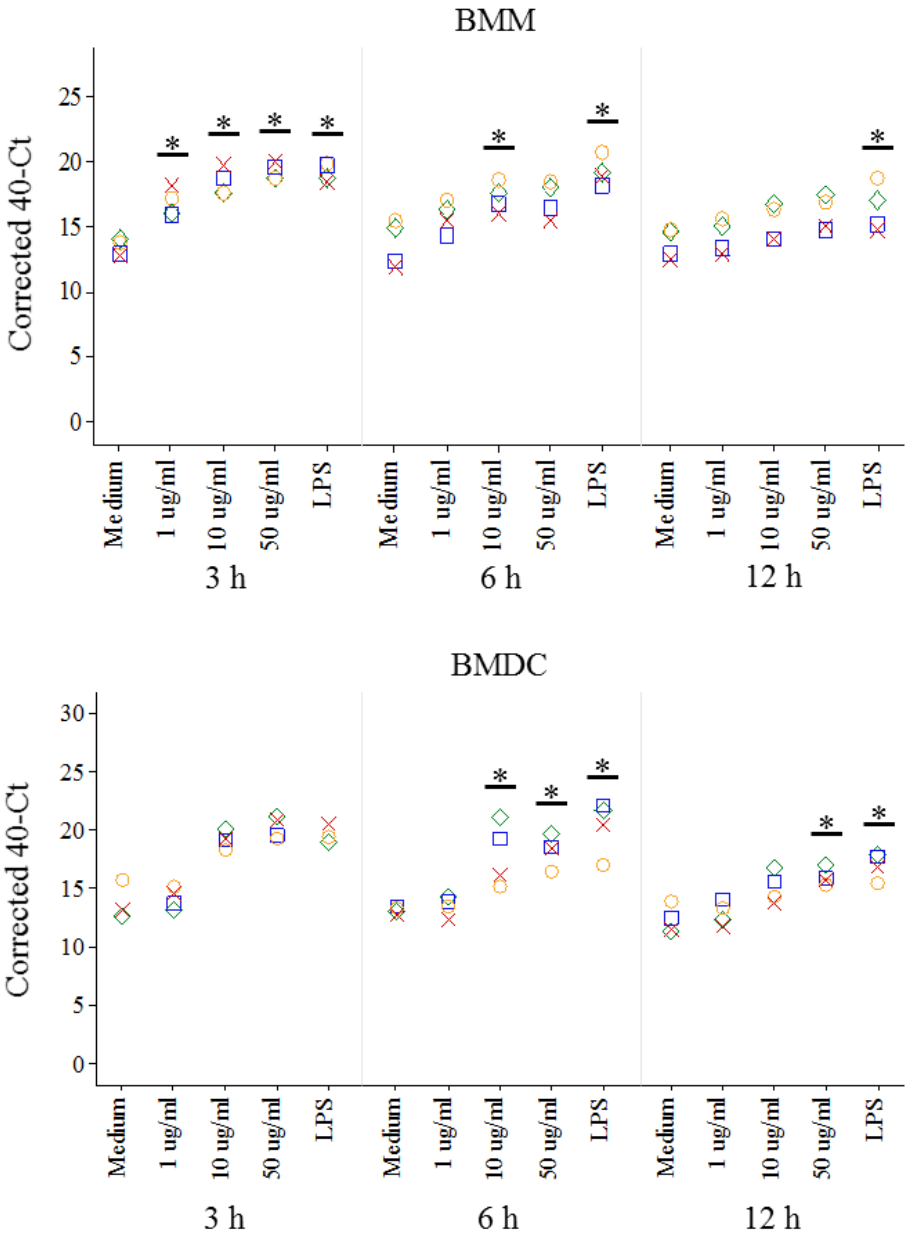

Figure 3-2: *IL6* mRNA expression in BMM and BMDC following stimulation with EtAg. BMM and BMDC were cultured until day 6 then incubated with medium alone (negative control), 1, 10 or 50 µg/ml of EtAg or 200 ng/ml of LPS (positive control) for 3, 6 and 12 hours. RNA was extracted and the expression of *IL6* mRNA measured by RT-qPCR. Data are presented as corrected 40-Ct values for four individual birds except for BMDC at 3 h where data are shown for three birds for medium and LPS-treated cultures. Individual colours denote data from individual birds. Asterisks indicate significant differences compared to medium controls at $p \leq 0.05$ according to the Mann-Whitney U test.

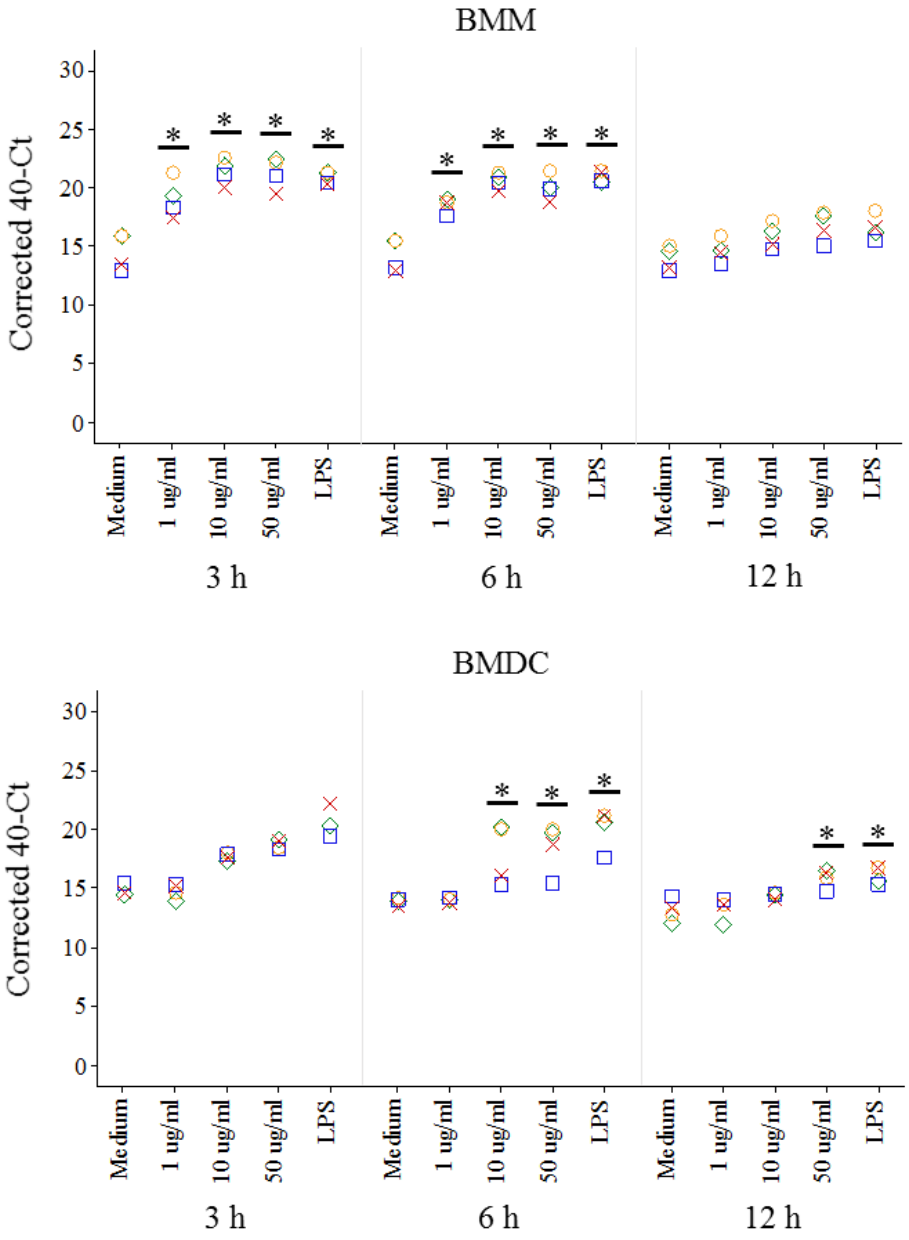

Figure 3-3: *NOS2* mRNA expression in BMM and BMDC following stimulation with EtAg. BMM and BMDC were cultured until day 6 then incubated with medium alone (negative control), 1, 10 or 50 µg/ml of EtAg or 200 ng/ml of LPS (positive control) for 3, 6 and 12 hours. RNA was extracted and the expression of *NOS2* mRNA measured by RT-qPCR. Data are presented as corrected 40-Ct values for four individual birds except for BMDC at 3 h where data are shown for three birds for medium and LPS-treated cultures. Individual colours denote data from individual birds. Asterisks indicate significant differences compared to medium controls at $p \leq 0.05$ according to the Mann-Whitney U test.

To investigate the role of BMM and BMDC in promoting a T helper response, the expression of Th1-associated cytokines *IFNG* (Figure 3-4) and *IL12A* (Figure 3-5) were measured. EtAg stimulation did not increase *IFNG* mRNA expression in BMM.

In BMDC, EtAg stimulation increased *IFNG* mRNA expression. Again, due to missing samples from one bird at 3 hps with medium and LPS, the upregulation of *IFNG* observed was not significant. At 6 and 12 hps, BMDC from two birds expressed consistently high *IFNG* mRNA regardless of treatment. The reasons for this are unclear; no bacterial contamination of these cultures was noted and the amplification curves of the RT-qPCR were normal.

Overall, EtAg stimulation did not increase *IL12A* mRNA expression in BMM. In BMDC, EtAg stimulation increased the expression of *IL12A* mRNA in a dose-dependent manner.

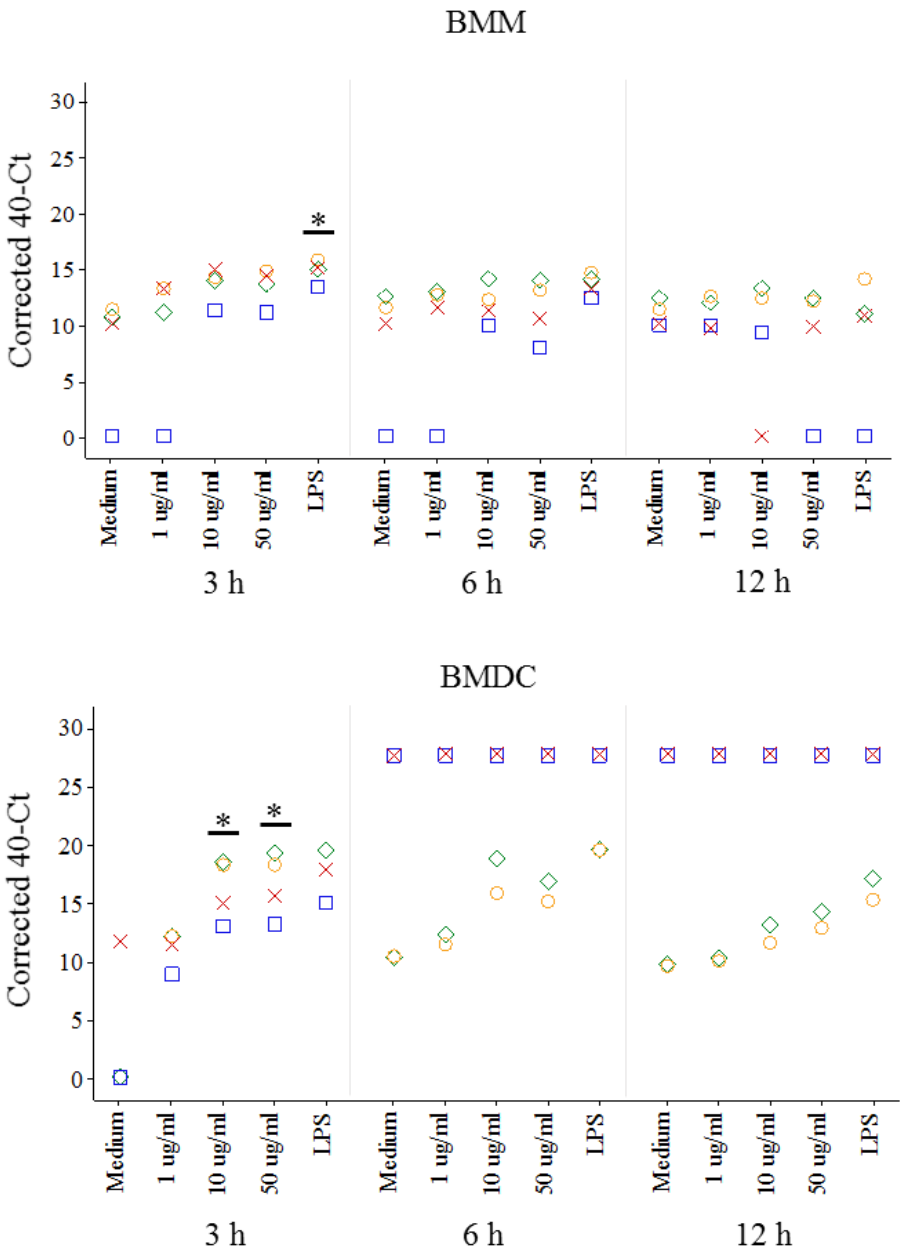

Figure 3-4: *IFNG* mRNA expression in BMM and BMDC following stimulation with EtAg.BMM and BMDC were cultured until day 6 then incubated with medium alone (negative control), 1, 10 or 50 µg/ml of EtAg or 200 ng/ml of LPS (positive control) for 3, 6 and 12 hours. RNA was extracted and the expression of *IFNG* mRNA measured by RT-qPCR. Data are presented as corrected 40-Ct values for four individual birds except for BMDC at 3 h where data are shown for three birds for medium and LPS-treated cultures. Individual colours denote data from individual birds. Asterisks indicate significant differences compared to medium controls at $p \leq 0.05$ according to the Mann-Whitney U test.

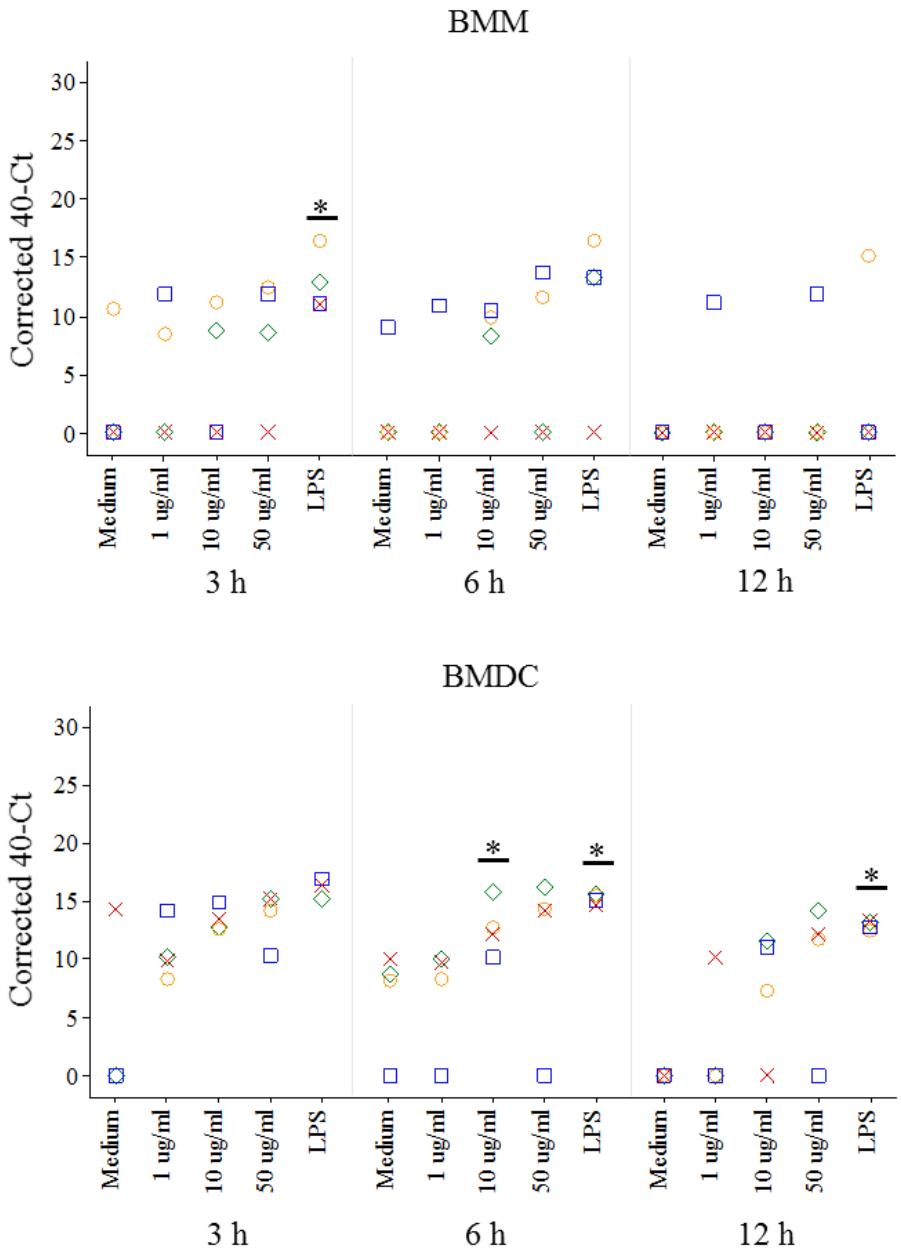

Figure 3-5: *IL12A* mRNA expression in BMM and BMDC following stimulation with EtAg. BMM and BMDC were cultured until day 6 then incubated with medium alone (negative control), 1, 10 or 50 µg/ml of EtAg or 200 ng/ml of LPS (positive control) for 3, 6 and 12 hours. RNA was extracted and the expression of *IL12A* mRNA measured by RT-qPCR. Data are presented as corrected 40-Ct values of four individual birds except for BMDC at 3 h where data are shown for three birds for medium and LPS-treated cultures. Individual colours denote data from individual birds. Asterisks indicate significant differences compared to medium controls at $p \leq 0.05$ according to the Mann-Whitney U test.

To investigate any potential Th2-promoting activity of BMM and BMDC, the expression of Th2-associated cytokines was measured. No change was observed in the expression of either *IL4* (Figure 3-6) or *TGFB4* mRNA (Figure 3-7) in either BMM or BMDC following stimulation with EtAg.

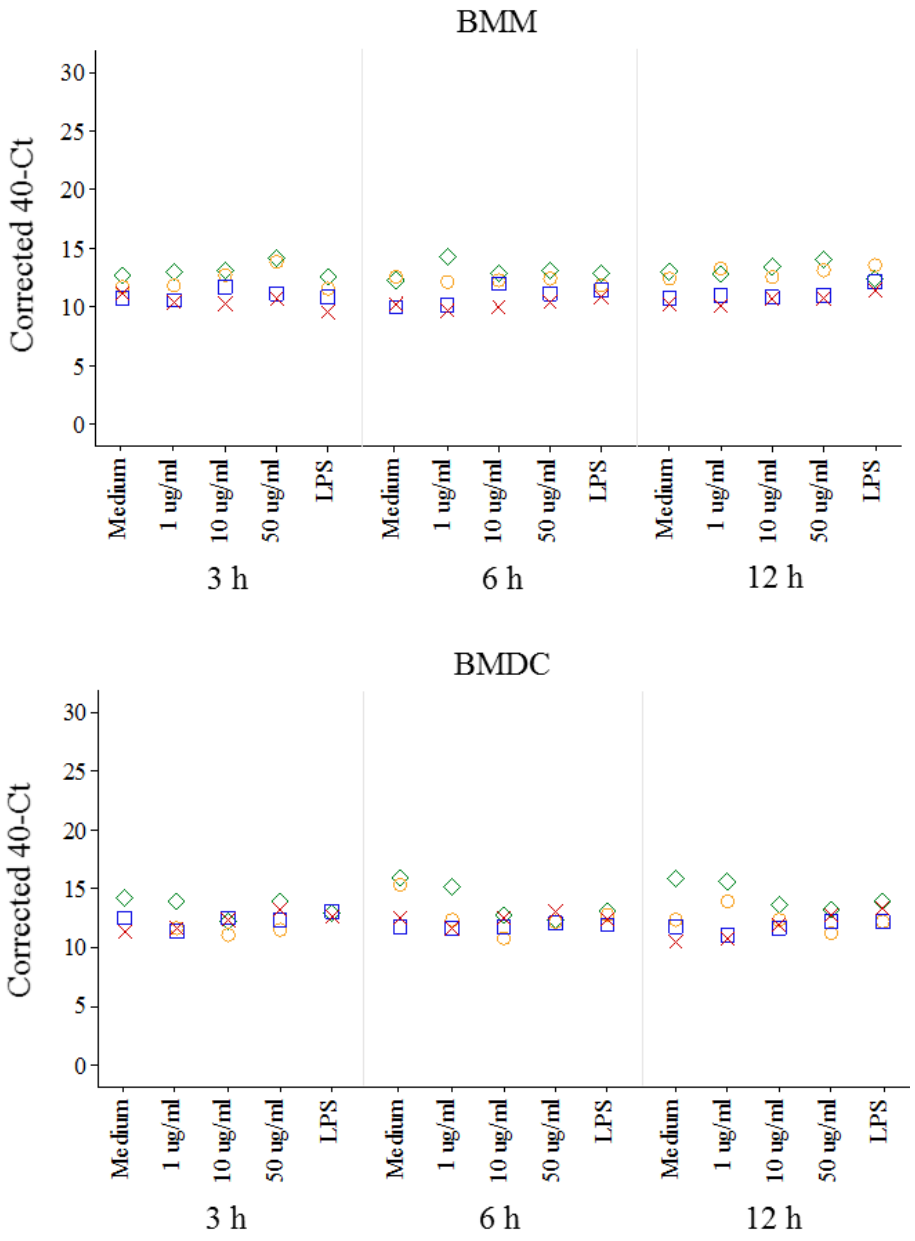

Figure 3-6: *IL4* mRNA expression in BMM and BMDC following stimulation with EtAg. BMM and BMDC were cultured until day 6 then incubated with medium alone (negative control), 1, 10 or 50 µg/ml of EtAg or 200 ng/ml of LPS (positive control) for 3, 6 and 12 hours. RNA was extracted and the expression of *IL4* mRNA measured by RT-qPCR. Data are presented as corrected 40-Ct values for four individual birds except for BMDC at 3 h where data are shown for three birds for medium and LPS-treated cultures. Individual colours denote data from individual birds. Asterisks indicate significant differences compared to medium controls at $p \leq 0.05$ according to the Mann-Whitney U test.

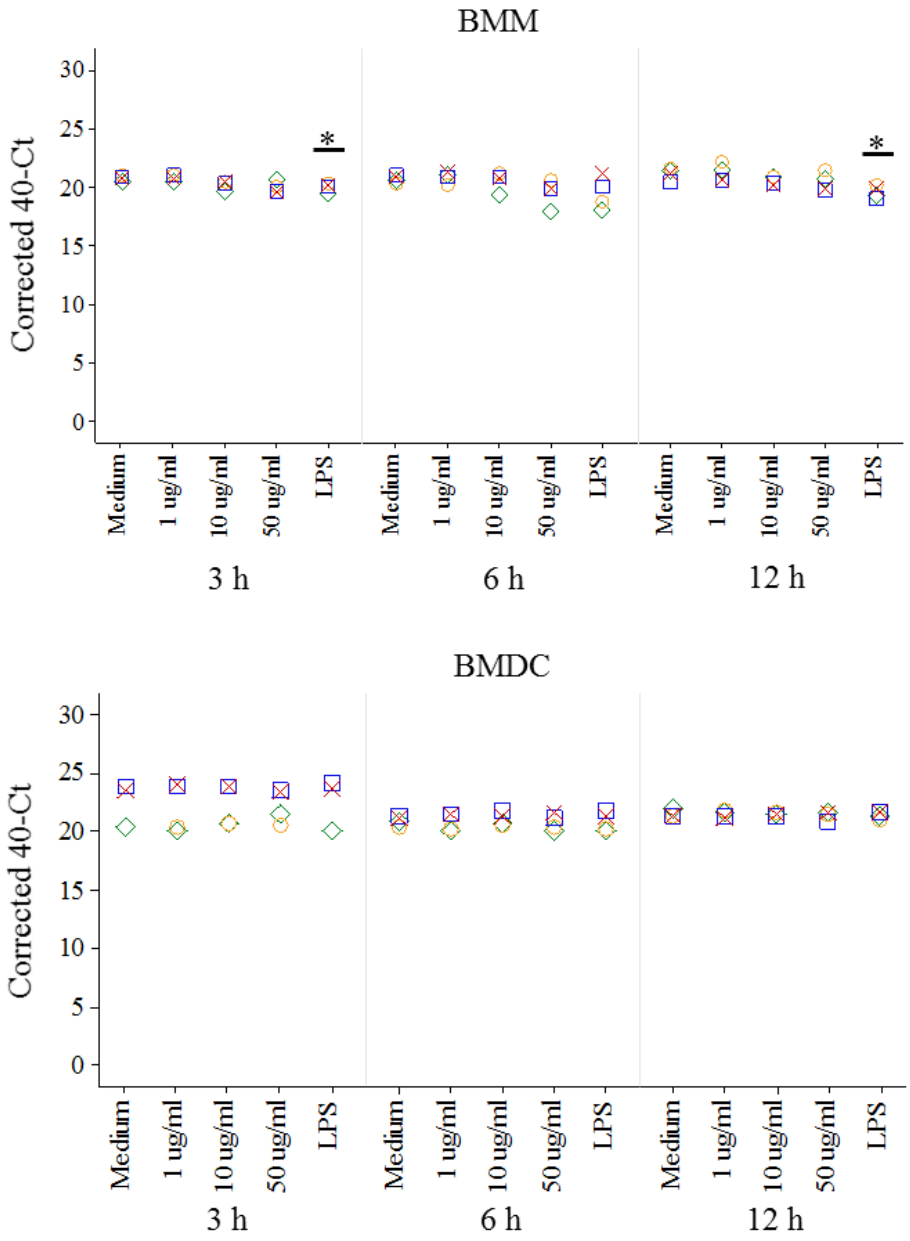

Figure 3-7: *TGFB4* mRNA expression in BMM and BMDC following stimulation with EtAg. BMM and BMDC were cultured until day 6 then incubated with medium alone (negative control), 1, 10 or 50 µg/ml of EtAg or 200 ng/ml of LPS (positive control) for 3, 6 and 12 hours. RNA was extracted and the expression of *TGFB4* mRNA measured by RT-qPCR. Data are presented as corrected 40-Ct values for four individual birds except for BMDC at 3 h where data are shown for three birds for medium and LPS-treated cultures. Individual colours denote data from individual birds. Asterisks indicate significant differences compared to medium controls at $p \leq 0.05$ according to the Mann-Whitney U test.

To investigate the regulatory activities of BMM and BMDC in response to EtAg, *IL10* mRNA was measured. In this chapter, the primers and probe set used to quantify *IL10* mRNA was IL10 (1) as given in Table 2-1 (Chapter 2). *IL10* mRNA expression did not change significantly in BMM following EtAg stimulation however did increase in BMM from some birds (Figure 3-8).

Following stimulation with EtAg, *IL10* mRNA expression in BMDC increased at 3 hps in a dose-dependent manner. Overall, BMM expressed more *IL10* mRNA than BMDC following stimulation with EtAg.

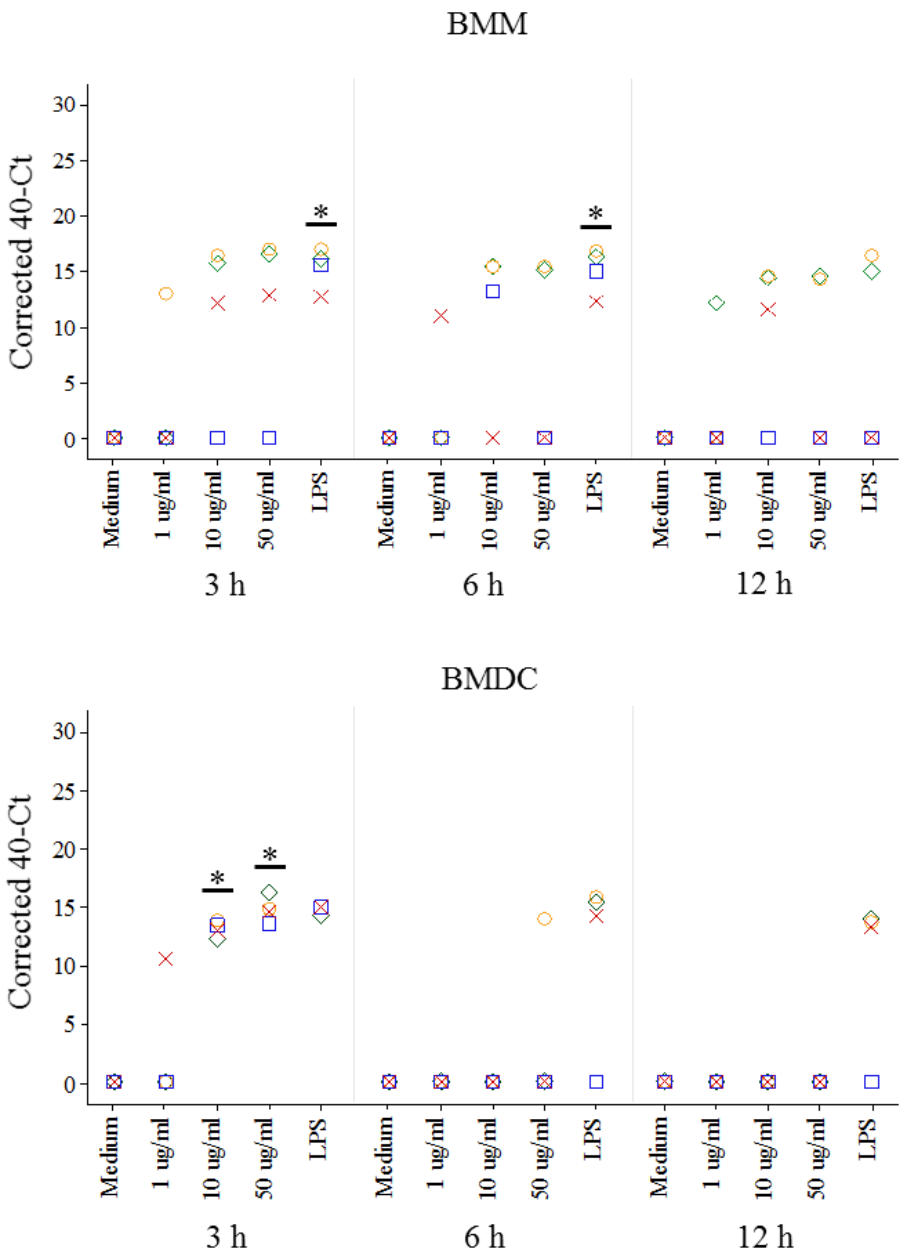

Figure 3-8: *IL10* mRNA expression in BMM and BMDC following stimulation with EtAg. BMM and BMDC were cultured until day 6 then incubated with medium alone (negative control), 1, 10 or 50 µg/ml of EtAg or 200 ng/ml of LPS (positive control) for 3, 6 and 12 hours. RNA was extracted and the expression of *IL10* mRNA measured by RT-qPCR. Data are presented as corrected 40-Ct values for four individual birds except for BMDC at 3 h where data are shown for three birds for medium and LPS-treated cultures. Individual colours denote data from individual birds. Asterisks indicate significant differences compared to medium controls at $p \leq 0.05$ according to the Mann-Whitney U test.

To determine if EtAg was toxic to the cells, the use of a live/dead cell stain was used. SYTOX® Blue, a dye which is able to enter dead cells and bind nucleic acids, was used to determine the proportion of live/dead cells within the $CD45^+$ cell population in EtAg-stimulated BMM cultures by FACS (Figure 3-9). BMM were cultured overnight with medium only, 1, 10 and 50 µg/ml of EtAg or 200 ng/ml of LPS. The forward scatter (FSC) vs side scatter (SSC) plot showed that the addition of EtAg to BMM cultures resulted in debris that lies both above the population observed for BMM and also within the same range of the BMM cell population (Figure 3-9A). Fewer live cells were also observed with increasing concentration of EtAg. The BMM cell population was gated on populations from the FSC vs. $CD45^+$ cells plots (Figure 3-9B) and as a result some EtAg debris (Figure 3-9B; EtAg only) was included in this population. The proportion of live and dead cells within the $CD45^+$ population was determined (Figure 3-9C). The proportion of dead cells increased with increasing concentration of EtAg, and although EtAg did appear to exhibit some toxicity towards the BMM, a large proportion of the dead cells observed was also expected to be due to the presence of EtAg debris.

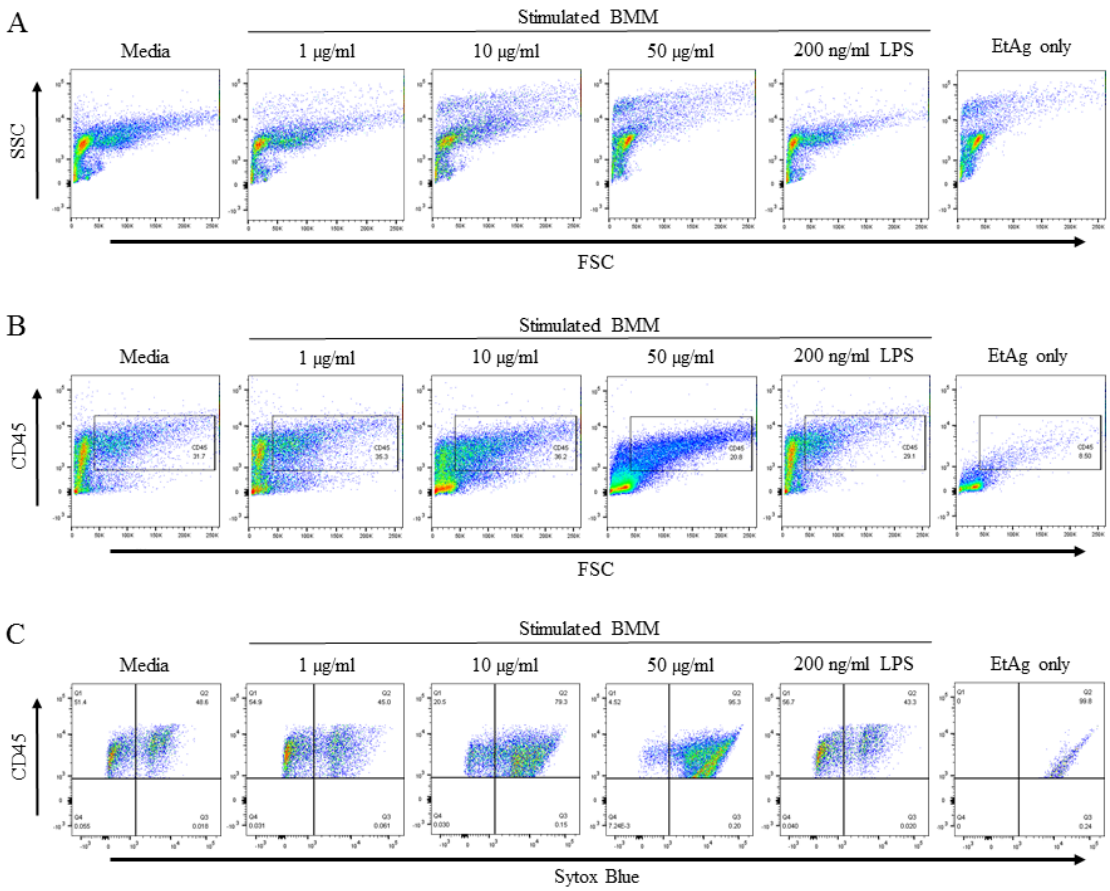

Figure 3-9: FACS analysis of BMM stimulated with EtAg. BMM were cultured from one Novogen Brown chicken until day 6 then stimulated with 1, 10 and 50 µg/ml of EtAg overnight, 200 ng/ml of LPS as a positive stimulation control and medium only as a negative control. To differentiate EtAg from BMM, BMM were stained with a mouse anti-chicken CD45 antibody (Clone LT40) and Sytox Blue to detect dead cells. Shown is the forward scatter (FSC) versus side scatter (SSC) of the entire BMM culture content (A), FSC versus $CD45^+$ cells with gating (indicated by rectangle) for $CD45^+$ cells (B) and the proportion of live/dead cells within the gated $CD45^+$ cell population shown by plots of Sytox Blue stained cells versus $CD45^+$ cells (C).

Although it appeared that EtAg is toxic to BMM, particularly at higher concentrations, it was also noted that, following culture with EtAg, BMM and BMDC were still adherent to the plates indicating that live cells were still present in the cultures indicating that a proportion of the cell death observed following FACS analysis may be due to the combination of EtAg stimulation and the harvesting and staining of the cells. To test if cells adherent to the plate following culture with EtAg were alive, BMM were stimulated overnight with medium, 1, 10 or 50 μg/ml of EtAg or 200 ng/ml of LPS and stained with propidium iodide (PI). Following stimulation with medium (Figure 3-10A) and 1 μg/ml of EtAg, (Figure 3-10B) many cells did not stain with PI and were adherent to the bottom of the plate indicating the presence of live cells. Following stimulation with 10 μg/ml of EtAg (Figure 3-10C), fewer live cells were observed although were still present. However, following stimulation with 50 μg/ml of EtAg (Figure 3-10D), very few live cells were adherent to the plate and more were stained with PI overall. Following LPS stimulation (Figure 3-10E), many of the cells were also found to be dead, however there was still a presence of high numbers of live cells adherent to the plate. This suggests that BMM were able to withstand EtAg up to concentrations of 10 μg/ml but at 50 μg/ml, EtAg becomes much more toxic to the cells, although there were still small numbers of live cells adherent to the plate at this concentration. As cells are lysed directly on the plate prior to RNA extraction, EtAg stimulated BMM cultures were suitable for RNA extraction and RT-qPCR analysis.

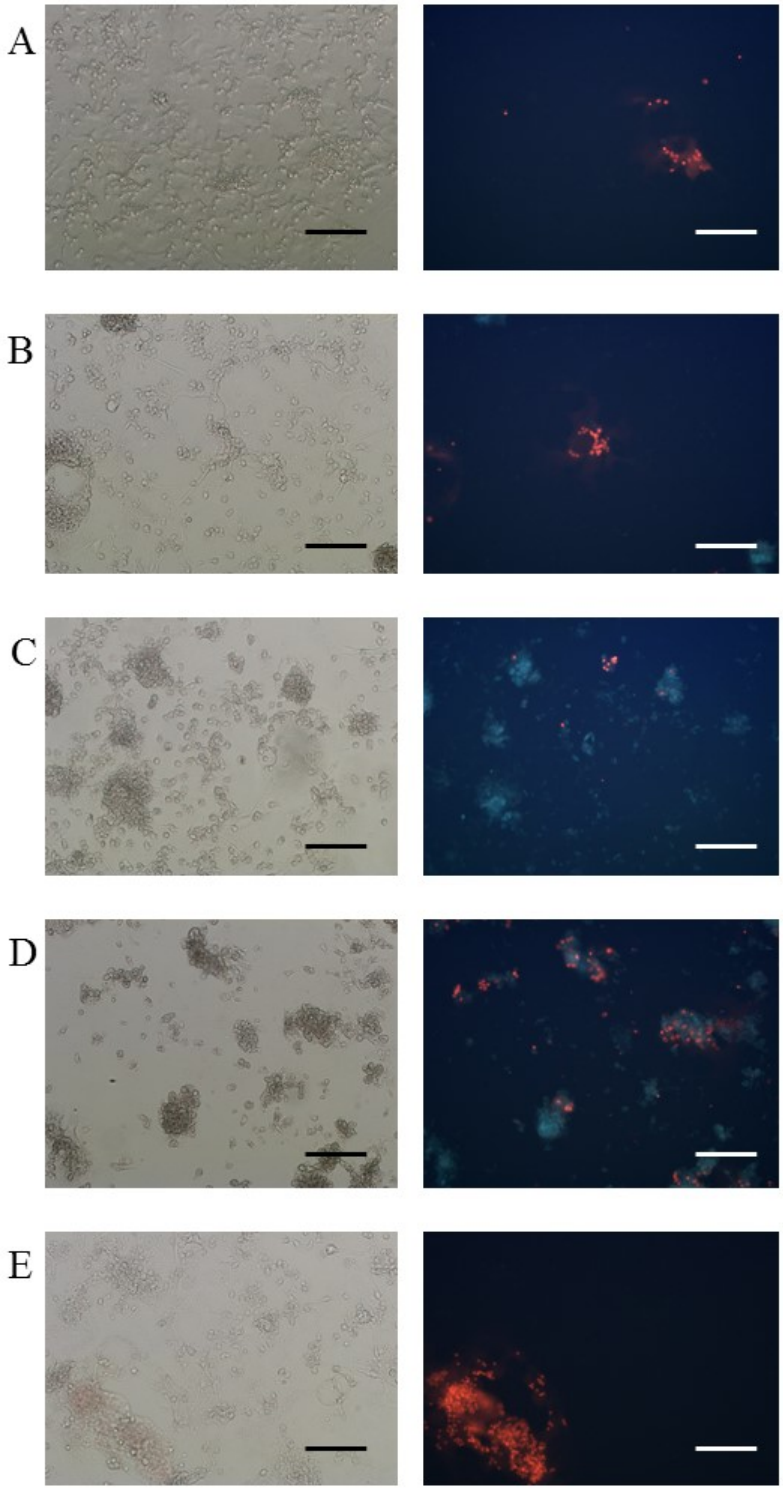

Figure 3-10: Propidium iodide (PI) stain of BMM stimulated with EtAg. BMM were cultured from one Novogen Brown chicken until day 6 then stimulated overnight with medium only (A), 1 µg/ml (B), 10 µg/ml (C), 50 µg/ml (D) of EtAg or 200 ng/ml LPS (E) and 30 µM of PI in BMM culture medium added. Bars represent 50 µm.

As LPS is a potent stimulator of BMM and BMDC, the presence of LPS in EtAg was determined using the Pierce™ LAL Chromogenic Endotoxin Quantitation Kit. At 50 µg/ml, EtAg contained LPS at a concentration of 0.03 ng/ml. At 10 and 1 µg/ml, the level of LPS in EtAg was below the detection limit of the assay (data not shown).

3.2.2 Response of BMM to vaccine candidates IMP1 and AMA1

Bacterial expression vectors (pET-32b; Novagen) containing the IMP1 and AMA1 genes of both *E. maxima* and *E. tenella* were supplied by Prof. D. Blake (RVC, London). IMP1 and AMA1 genes from *E. tenella* and *E. maxima* were successfully sub-cloned from the pET32b bacterial expression vector into the pSecTag2 A mammalian expression vector using the methods outlined in section 2.1. The resulting plasmid sequences were confirmed using the Sanger sequencing method by Edinburgh Genomics. The purpose of sub-cloning these genes into a mammalian expression vector was to produce recombinant IMP1 and AMA1 in a mammalian expression system, eliminating the possibility of LPS contamination that would have arisen from use of a bacterial expression system. Proteins produced in bacteria also undergo different post-translational modifications to those in eukaryotic cells, and by using a mammalian expression system to produce recombinant IMP1 and AMA1, post-translational modifications would more closely reflect that of *Eimeria*.

Recombinant EmIMP1, EtIMP1 and EtAMA1 were successfully expressed in COS-7 cells and detected in supernatants by dot blot. EmAMA1 was not detected in either supernatants or COS-7 cell lysates following the DEAE-dextran method of transfection with the pSecTag2 A-EmAMA1 plasmid, however was detectable in

HEK293T cell supernatant transfected using the PEI method of transfection (Figure 3-11A).

To determine the concentrations of recombinant protein in transfected cell supernatants, a LI-COR western blot was performed (Figure 3-11B). A recombinant ovine prion protein (OvPrP; Whyte *et al.* (2003)) was kindly provided by Dr A. Gill (Roslin Institute, UK) and used to generate a standard curve against which the approximate concentrations of recombinant *Eimeria* proteins were calculated (Table 3-1).

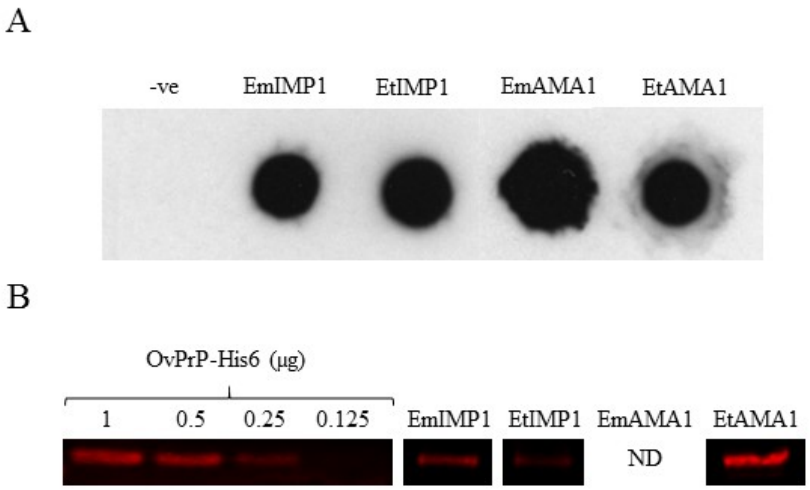

Figure 3-11: Detection and quantification of recombinant *Eimeria* proteins in mammalian cell lines as detected by His-tag. Expression of recombinant EmIMP1 (75 kDa), EtIMP1 (70 kDa) and EtAMA1 (68 kDa) in COS-7 cells and EmAMA1 (59 kDa) in HEK293T cells was detected by dot blot. Mock-transfected COS-7 supernatant was used as a negative control (A). The concentration of recombinant proteins in supernatants was measured by LI-COR western blot. OvPrP (17.5 kDa) of known concentration was used to produce a standard curve against which the concentration of recombinant *Eimeria* proteins was calculated (B). ND; not detected.

Table 3-1: Concentration of recombinant *Eimeria* proteins as determined by LI-COR western blot. Transfected COS-7 and HEK293T cell culture supernatants containing recombinant *Eimeria* proteins were ran alongside known concentrations of recombinant OvPrP and the staining intensity of each band measured using Image Studio Lite version 5.0. The concentration of recombinant proteins present in supernatants was calculated against a standard curve generated from the OvPrP concentrations.

Recombinant Protein	**Concentration (µg/ml)**
EmIMP1	79.2
EtIMP1	34.0
EmAMA1	Not detectable by western blot
EtAMA1	214.5

BMM were stimulated with recombinant EmIMP1, EtIMP1, EmAMA1 and EtAMA1 for 3, 6 and 24 h. EmAMA1 stimulation increased *IL6* mRNA expression in BMM (Figure 3-12).

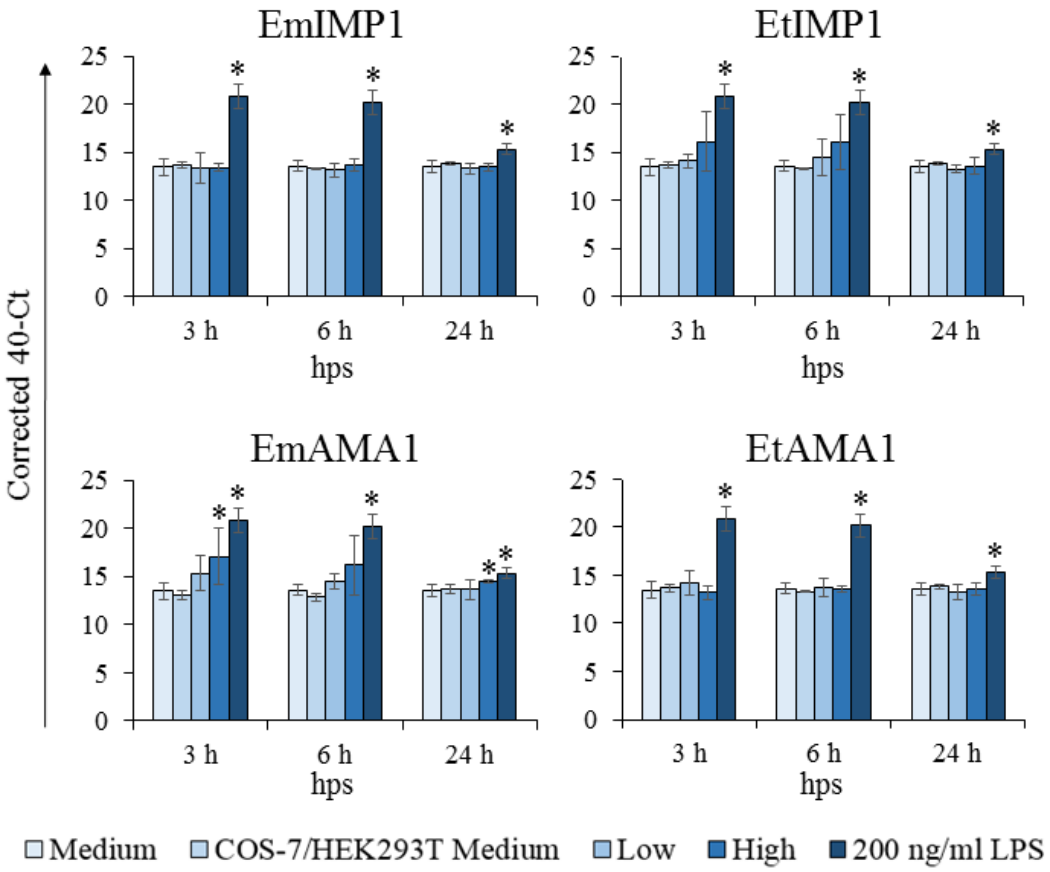

Figure 3-12: *IL6* mRNA expression in BMM stimulated with *Eimeria* recombinant proteins. BMM were cultured until day 6 then stimulated with two concentrations of recombinant EmIMP1, EtIMP1, EmAMA1 or EtAMA1, medium only (negative control), mock-transfected COS-7 or HEK293T medium (negative control) or 200 ng/ml LPS (positive control) for 3, 6 and 24 h. RNA was extracted and *IL6* mRNA measured by RT-qPCR. For EmIMP1, EtIMP1 and EtAMA1 the low concentration was 1 µg/ml and the high was 10 µg/ml. The concentration of recombinant EmAMA1 was not determined and therefore a dilution of 1 in 4 was used for the low concentration and 1 in 40 for the high concentration. Data are presented as the mean corrected 40-Ct of three biological replicates ± SD. Asterisks indicate significant differences compared to media controls at $p \leq 0.05$ according to the Mann-Whitney U test.

The expression of *NOS2* mRNA was also measured. EtIMP1 and EmAMA1 stimulation increased *NOS2* mRNA expression in BMM (Figure 3-13).

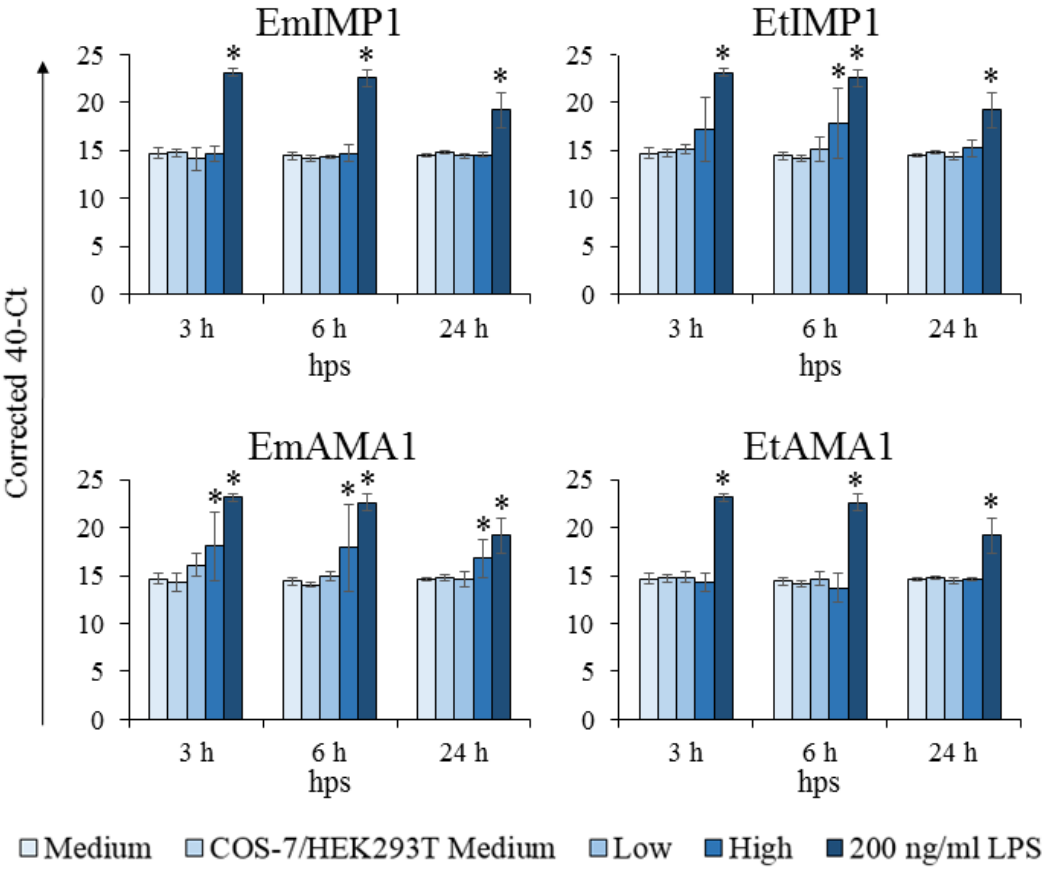

Figure 3-13: *NOS2* mRNA expression in BMM stimulated with *Eimeria* recombinant proteins. BMM were cultured until day 6 then stimulated with two concentrations of recombinant EmIMP1, EtIMP1, EmAMA1 or EtAMA1, medium only (negative control), mock-transfected COS-7 or HEK293T medium (negative control) or 200 ng/ml LPS (positive control) for 3, 6 and 24 h. RNA was extracted and *NOS2* mRNA measured by RT-qPCR. For EmIMP1, EtIMP1 and EtAMA1 the low concentration was 1 µg/ml and the high was 10 µg/ml. The concentration of recombinant EmAMA1 was not determined and therefore a dilution of 1 in 4 was used for the low concentration and 1 in 40 for the high concentration. Data are presented as the mean corrected 40-Ct of three biological replicates ± SD. Asterisks indicate significant differences compared to media controls at $p \leq 0.05$ according to the Mann-Whitney U test.

3.2.3 Recognition of *Eimeria* by chicken TLRs

Before TLR recognition of *Eimeria* antigens was investigated, the expression of TLRs in BMM and BMDC was first confirmed by RT-qPCR. The expression of TLR1LA, TLR1LB, TLR2A, TLR2B and TLR21 was measured in BMM and BMDC stimulated with medium, EtAg and LPS (Figure 3-14). All TLRs measured were expressed in both BMM and BMDC but overall levels of expression did not change following stimulation with either EtAg or LPS.

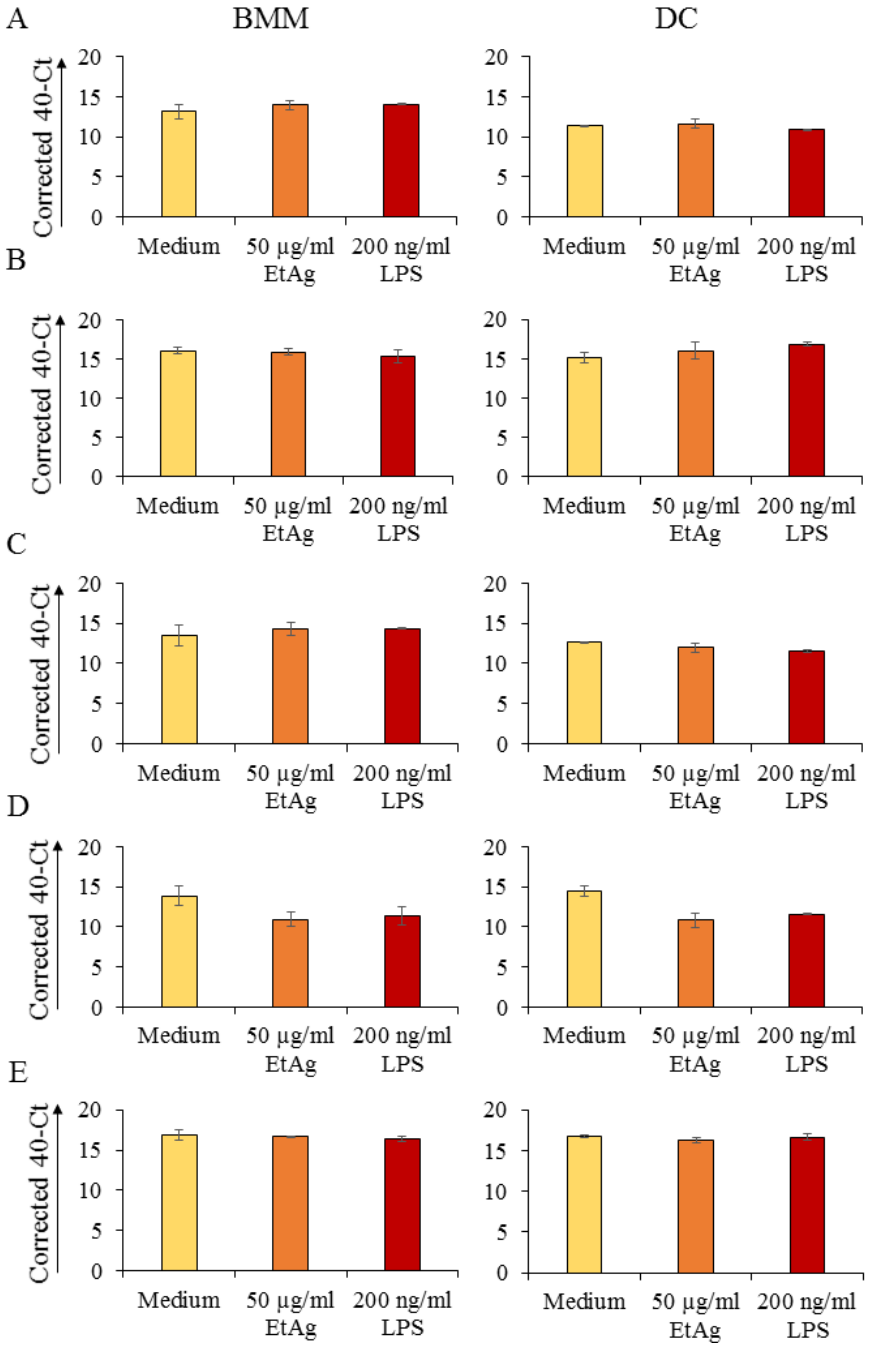

Figure 3-14: Expression of TLR mRNA in BMM and BMDC following stimulation with EtAg. BMM and BMDC were cultured until day 6 then cultured with medium alone (negative control), 50 µg/ml EtAg or 200 ng/ml LPS for 6 hours. RNA was extracted and 150 ng of RNA was pooled between two sets of two birds in each group and expression of *TLR1LA* (A), *TLR1LB* (B), *TLR2A* (C), *TLR2B* (D) and *TLR21* (E) measured by RT-qPCR. Data are presented as the mean corrected 40-Ct of the two samples of each group ±SEM of triplicate wells.

To elucidate which chicken TLRs may be important in host recognition of various *Eimeria* PAMPs, a HEK293T-SEAP reporter gene assay was performed to determine if recombinant EmIMP1, EtIMP1, EmAMA1, EtAMA1 or EtAg were recognised by TLR1LB/2A or TLR1LB/2B heterodimers or TLR21.

Increased OD values were observed in supernatants of cells expressing TLR1LB/2A heterodimers and stimulated with neat supernatants containing EtIMP1, EtAMA1 and EmAMA1 indicating that these recombinant proteins can be recognised by TLR1LB/2A heterodimers (Figure 3-15).

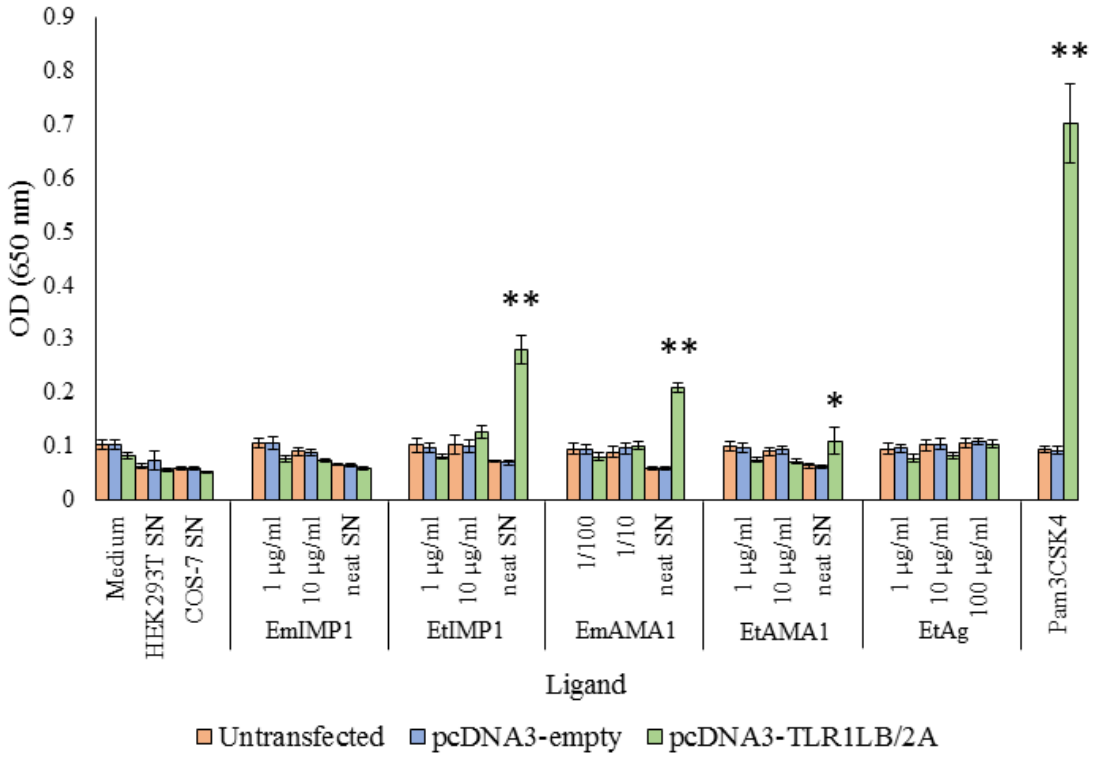

Figure 3-15: Recognition of recombinant *Eimeria* proteins IMP1 and AMA1 and EtAg by TLR1LB/2A heterodimers. HEK293T-SEAP cells were either transfected with pcDNA3-TLR1LB-YFP and pcDNA3-TLR2A-YFP, pcDNA3-empty vector or left untransfected then stimulated with 1 µg/ml, 10 µg/ml or neat (undiluted) COS-7 or HEK293T supernatant (SN) containing recombinant IMP1 and AMA1 from both *E. tenella* (Et) and *E. maxima* (Em) or EtAg. The concentration of recombinant EmAMA1 was not determined therefore dilutions of 1 in 10 and 1 in 100 were used. As a positive control Pam3CSK4 was used at 100 ng/ml. As negative controls, DMEM medium (2% FBS), mock-transfected HEK293T supernatant and mock-transfected COS-7 SN were used. Results shown are means of triplicate wells from three technical replicates ± SEM. Single asterisk indicates significance at $p \leq 0.05$ and double asterisks at $p < 0.001$ compared to medium, HEK293T and COS-7 supernatant stimulated wells according to the Mann Whitney U test.

No increases to OD values in supernatants of HEK293T cells expressing TLR1LB/2B heterodimers were observed with any of the *Eimeria* antigens tested indicating that TLR1LB/2B heterodimers do not recognise these *Eimeria* antigens (Figure 3-16).

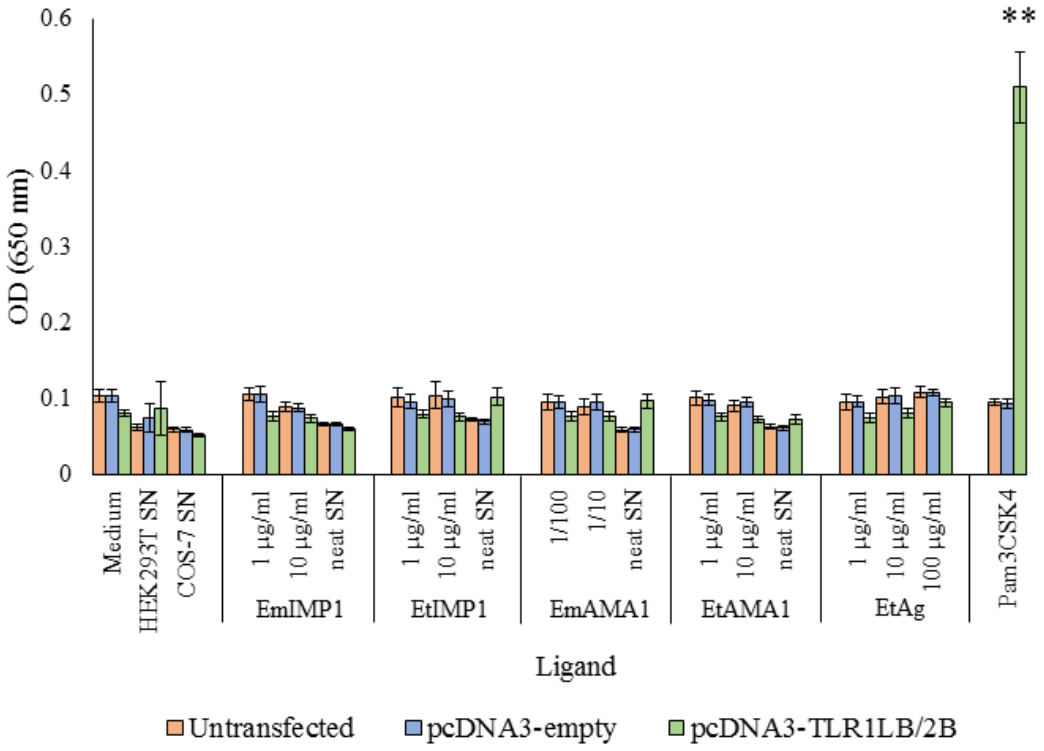

Figure 3-16: Recognition of recombinant *Eimeria* proteins IMP1 and AMA1 and EtAg by TLR1LB/2B heterodimers. HEK293T-SEAP cells were either transfected with pcDNA3-TLR1LB-YFP and pcDNA3-TLR2B-YFP, pcDNA3-empty vector or left untransfected then stimulated with 1 µg/ml, 10 µg/ml or neat (undiluted) COS-7 or HEK293T supernatant (SN) containing recombinant IMP1 and AMA1 from both *E. tenella* (Et) and *E. maxima* (Em) or EtAg. The concentration of recombinant EmAMA1 was not determined therefore dilutions of 1 in 10 and 1 in 100 were used. As a positive control Pam3CSK4 was used at 100 ng/ml. As negative controls, DMEM medium (2% FBS), mock-transfected HEK293T supernatant and mock-transfected COS-7 supernatant were used. Results shown are means of triplicate wells from three technical replicates ± SEM. Double asterisks indicate significance at $p<0.001$ compared to medium, HEK293T and COS-7 supernatant stimulated wells according to the Mann Whitney U test.

No increases to OD values in supernatants of HEK293T cells expressing TLR21 were observed with any of the *Eimeria* antigens tested indicating that TLR21 does not recognise these antigens (Figure 3-17).

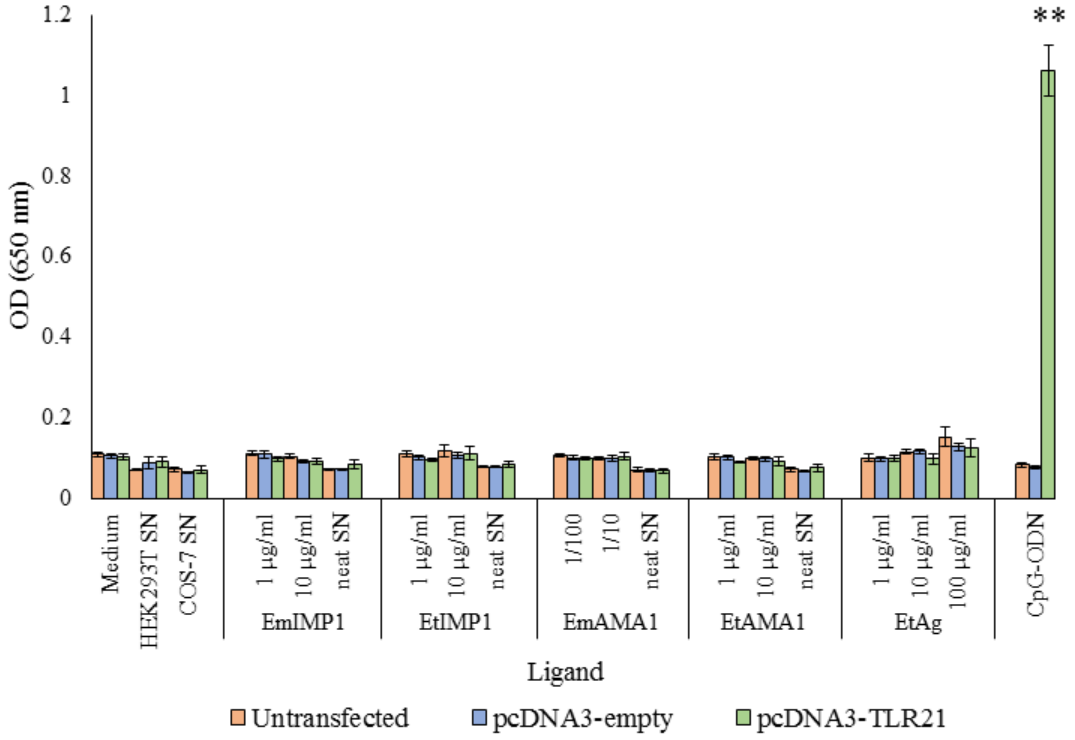

Figure 3-17: Recognition of recombinant *Eimeria* proteins IMP1 and AMA1 and EtAg by TLR21. HEK293T-SEAP cells were either transfected with pcDNA3-TLR21-YFP, pcDNA3-empty vector or left untransfected then stimulated with 1 µg/ml, 10 µg/ml or neat (undiluted) COS-7 or HEK293T supernatant (SN) containing recombinant IMP1 and AMA1 from both *E. tenella* (Et) and *E. maxima* (Em) or EtAg. The concentration of recombinant EmAMA1 was not determined therefore dilutions of 1 in 10 and 1 in 100 were used. As a positive control, CpG-ODN was used at 25 µg/ml. As negative controls, DMEM medium (2% FBS), mock-transfected HEK293T supernatant and mock-transfected COS-7 supernatant were used. Results shown are means of triplicate wells from three technical replicates ± SEM. Double asterisks indicate significance at $p<0.001$ compared to medium, HEK293T and COS-7 supernatant stimulated wells according to the Mann Whitney U test.

3.3 Discussion

Previous studies investigated the role of the HD11 and HTC chicken macrophage-like cell lines and their response to *Eimeria* and *Eimeria* antigens however the responses of primary cells, chicken BMM and BMDC, have not been investigated. To assess the role of APC in mediating the inflammatory response during *Eimeria* infection, BMM and BMDC were stimulated with EtAg and proinflammatory, Th1 and Th2 driving cytokines were measured by RT-qPCR. In response to EtAg, BMM and BMDC both increased mRNA expression of proinflammatory cytokine genes including *IL1B*, *IL6* and *NOS2* in a dose-dependent manner. BMM and BMDC appeared to have similar capacities to promote inflammatory responses to EtAg. Other studies have examined the responses of macrophage cell lines to *Eimeria* antigens. The HTC cell line expressed more *NOS2* and *IL1B* mRNA in response to *E. tenella* surface antigens (SAGs) and *IL1B*, *IL6* and *IL18* mRNA in response to *E. maxima*, *E. tenella* and *E. acervulina* sporozoites (Chow *et al.*, 2011). In another study, the HD11 macrophage-like cell line increased NO production following stimulation with *E. tenella* sporozoites (Lillehoj *et al.*, 2004).

IFNG and *IL12A* mRNA were measured to assess the potential of BMM and BMDC in driving Th1 responses. EtAg stimulation did not increase *IFNG* or *IL12A* expression in BMM but did increase *IFNG* and *IL12A* expression in BMDC from some birds. Previous studies have also shown DCs to have a role in the IFNγ/Th1 response to *Eimeria.* Immunisation of chickens with DC-derived exosomes containing *E. maxima*, *E. tenella* or *E. acervulina* antigens resulted in increased numbers of IFN-γ-producing cells in the spleen, CT and PP. In addition, increased weight gains and reduced oocyst shedding were observed in the birds following

challenge (del Cacho *et al.*, 2012). In another study, *E. acervulina* 3-1E profilin stimulated IL-12p70 production in murine DCs (Rosenberg *et al.*, 2005) further adding to the case that DCs are capable of initiating Th1 responses to *Eimeria*. During *Eimeria* infection, IFN-γ is thought to be important not only in initiating the Th1 response but in reducing sporozoite replication. Pre-treatment of BMM with recombinant IFN-γ resulted in a reduced ability of sporozoites to replicate within macrophages (Dimier *et al.*, 1998). The results of this study indicate that BMDC have the capacity to promote a Th1 response following stimulation with *Eimeria* antigens and indicate that DC can be more efficient in driving the Th1 response during *Eimeria* infection than macrophages. However, a previous study found that in response to *E. tenella* merozoite lysate, the HTC cell line increased expression of *IL12* and *IFNG* mRNA transcripts (Chow *et al.*, 2011). Another study demonstrated that the same cell line increased *IFNG* mRNA transcripts in response to *E. tenella* sporozoites however, decreased *IFNG* production in response to *E. maxima* and *E. tenella* sporozoites was also observed (Dalloul *et al.*, 2007).

As expected, EtAg did not stimulate the expression of Th2-associated cytokines *TGFB4* and *IL4* in either BMM or BMDC in response to EtAg. EtAg stimulated the expression of *IL10* mRNA in BMM, although not significantly, and in BMDC only at 3 hps. The results indicate that both macrophages and DCs produce IL-10 in response to *Eimeria* antigens, but macrophages are likely the main cell type to produce IL-10 during *Eimeria* infection (Rothwell *et al.*, 2004). The HTC cell line increased *IL10* mRNA expression in response to *E. tenella* merozoite lysate (Chow *et al.*, 2011) and together, with the findings of this study, support a regulatory role for macrophages during *Eimeria* infection.

The concentration of LPS in 50 µg/ml of EtAg was 0.03 ng/ml. LPS is a potent stimulator of BMM and BMDC, which respond to LPS in a dose-dependent manner (Sutton, 2014). The response of these cells to concentrations of LPS below 1 ng/ml has not been studied, however based on the dose-dependent manner to which these cells respond, it is unlikely that the LPS concentration in EtAg is sufficient to cause the increases in cytokine mRNA observed in this study. However, EtAg at 50 µg/ml and LPS at 200 ng/ml is toxic to the cells, therefore the results of BMM and BMDC stimulated with these concentrations of EtAg and LPS should be interpreted with caution. Previous studies have used LPS at concentrations of 200 ng/ml to stimulate chicken BMDC (Wu *et al.*, 2010) and 500 ng/ml to stimulate chicken BMM (Wu *et al.*, 2016). The results of this study suggest that the concentration of LPS with which BMM and BMDC are stimulated should be optimised for both maximal responses and minimal toxicity before future experiments are performed.

Recombinant IMP1 and AMA1 were successfully sub-cloned from the bacterial expression vector (pET32b) to the mammalian expression vector (pSecTag2A) and expression was detected in either transfected COS-7 or HEK293T cell culture supernatants by dot blot. The concentration of each protein in supernatant was determined by LI-COR western blot. However, the size of the OvPrP was much smaller (approx. 19 kDa; Whyte *et al.* (2003)) in comparison with that of the *Eimeria* recombinant proteins IMP1 and AMA1 (ranging from 59-75 kDa; (Jiang *et al.*, 2012; Jenkins *et al.*, 2015)). The discrepancy between the size of the protein standard and the recombinant proteins likely contributed towards the high concentration values obtained which were likely to be overestimates. To overcome this issue, proteins were quantified on the same western blot so as to achieve concentration values

relative to one another, ensuring that BMM were stimulated with similar concentrations of each protein. As the concentration of each protein is likely an overestimate, BMM were stimulated with high concentrations (10 and 1 μg/ml) of each recombinant protein. In the case of EmAMA1 where a concentration was not obtained but the protein was present in HEK293T cell supernatant, dilutions of 1 in 4 and 1 in 40 of EmAMA1 were used.

These results show that BMM are capable of responding in a proinflammatory fashion to recombinant EtIMP1 and EmAMA1 by upregulation of *IL6* and *NOS2* mRNA, indicating that macrophages could have a role in recognising these proteins in response to vaccination and in doing so facilitate inflammatory responses. No responses were observed by BMM in response to EmIMP1 or EtAMA1. It is possible that this is due to the over-estimation of protein concentrations and that sufficient protein was not present to elicit a response from the BMM. To improve upon these results, purification of the recombinant proteins would be necessary and would allow for the further characterisation of BMM responses to these proteins and the capacity of BMM to produce Th-promoting cytokines could be examined.

The roles of APC during *Eimeria* infection have not been fully characterised. During infection, *Eimeria* sporozoites enter the gut epithelium at the villi tips. Sporozoites then travel towards the crypts where replication occurs and schizonts are formed. The route by which sporozoites travel from the villi tips to the crypts has not been determined but it has been suggested that macrophages are involved in phagocytosis and transport of sporozoites to the crypt regions in the gut (Challey *et al.*, 1959; Rose *et al.*, 1977). Another study found that the majority of sporozoites were present in

host cells on their journey from the villus epithelium to the crypts, but that these cells were not macrophages but IELs (Lawn *et al.*, 1982). Vervelde *et al.* (1996) found that more sporozoites were found next to or within macrophages in naïve chickens compared with immune chickens and Cornelissen *et al.* (2009) found that the numbers of macrophages in the duodenum, jejunum and caecum of broilers increased following *E. acervulina*, *E. maxima* and *E. tenella* infection respectively and Swinkels (2008) found increased macrophages in the jejunum of broilers following *E. maxima* and *E. acervulina* infection. Other *in vivo* studies have shown that macrophage numbers did not increase following *E. maxima* and *E. tenella* infection (This Thesis, Chapter 3) and *E. acervulina* infection (Velkers *et al.*, 2010) in broilers. However, following *E. falciformis* infection in mice, increased numbers of macrophages were observed in the caecum (Schmid *et al.*, 2014) and during *E. bovis* infection in calves, macrophages infiltrate the gut following primary infection and, to an even greater extent, following secondary infection (Taubert *et al.*, 2009).

This chapter also aimed to identify which chicken TLRs recognised EmIMP1, EtIMP1, EmAMA1, EtAMA1 and EtAg by means of a SEAP reporter gene assay. When stimulated with *Eimeria* antigens, HEK293T-SEAP cells expressing TLR1LB/2B heterdimers or TLR21 did not increase activation of NF-κB and so these TLRs are unlikely involved in recognition of *Eimeria* IMP1 or AMA1. NF-κB was activated in HEK293T-SEAP cells expressing chicken TLR1LB/2A heterodimers when stimulated with EtIMP1, EtAMA1 and EmAMA1 indicating that these proteins could be involved in host recognition of *E. maxima* and *E. tenella*. Upon stimulation with TLR2 ligands peptidoglycan and Pam3CSK4, oxidative burst, degranulation and expression of *IL1B*, *IL6* and *IL8* mRNA increased in chicken

heterophils (Kogut *et al.*, 2005a). In mammals, TLR1/2 heterodimers present on mice BMM recognise GPI from *Plasmodium falciparum* and in response increase production of proinflammatory IL-6 and nitrite (Zhu *et al.*, 2011) and stimulation of murine BMM with TLR2 ligand Pam3CSK4 can induce *IL1B* and *IL18* mRNA expression (Zhu *et al.*, 2017). These studies, together with the finding that EtIMP1, EtAMA1 and EmAMA1 can be recognised by chicken TLR1LB/2A heterodimers, support a role for TLR1LB/2A in recognising *Eimeria* and inducing the production of proinflammatory mediators in APC.

Stimulation of TLR21-transfected HEK293T-SEAP cells with EtAg did not result in the activation of NF-κB. CpG ODN is the natural ligand for TLR21 and EtAg is expected to contain CpG DNA therefore the reasons for its failure to activate NF-κB in HEK293T-SEAP cells expressing TLR21 are unclear. One possibility is the sensitivity of the assay as even in the CpG ODN positive control, a high concentration was required to stimulate NF-κB activation and drive SEAP expression. This, combined with a possible low concentration of CpG DNA in EtAg, would result in failure of TLR21 to detect CpG DNA in this context. Chicken TLR21 is the functional homolog of mammalian TLR9 which plays an important role in the recognition of other protozoans. In *T. gondii* infection, $TLR9^{-/-}$ mice fail to mount Th1-responses and display sub-optimal IFNγ production (Minns *et al.*, 2006) and TLR9 can also recognise *P. falciparum* hemozoin, a byproduct of the digestion of haemaglobin by the parasite (Coban *et al.*, 2005). Although hemazoin is not produced by *Eimeria*, this demonstrates the plasticity of TLRs to recognise ligands outwith their natural repertoire.

TLRs 1LA, 1LB and 2 are up-regulated in caecal IELs in response to *E. tenella* infection suggesting a role in early recognition of the parasite (Zhang *et al.*, 2012). Mammalian TLR2 is able to recognise protozoan GPI which is expressed on protozoan parasite surfaces (Campos *et al.*, 2001). As chicken TLR2A and TLR2B are similar to mammalian TLR2, chicken TLR1/2 heterodimers containing TLR2A or 2B are candidate TLRs in the recognition of GPI-anchored *Eimeria* proteins. GPI-anchored surface antigens (SAG) from *E. tenella* induced inflammatory responses (increased nitrite production and expression of *NOS2* and *IL1B* mRNA) in the HTC macrophage cell line (Chow *et al.*, 2011). These findings indicate that macrophages can recognise GPI-anchored *Eimeria* surface proteins and in response increase the production of proinflammatory mediators. In heterophils and monocyte-derived macrophages, TLR4 and TLR15 are both upregulated following stimulation with live and heat killed *E. tenella* sporozoites (Zhou *et al.*, 2013) suggesting these TLRs are involved in recognising *Eimeria* sporozoites. Murine TLR11 can recognise toxofilin, the *T. gondii* homolog of profilin. Profilin is an actin-binding protein, which is highly conserved across apicomplexans including *Eimeria* and is expressed throughout most stages of the life cycle (Fetterer *et al.*, 2004; Lee *et al.*, 2013). Recognition of toxofilin by DCs via TLR11 induced $CD4^+$ T cell-mediated immunity against the pathogen by increasing IL-12p40 production in DCs (Yarovinsky *et al.*, 2005). Additionally, TLR11 can also recognise FliC, a component of flagellin, from *Salmonella typhimurium* and *E. coli* (Mathur *et al.*, 2012). A chicken homolog of TLR11 has not been identified, but given the ability of murine TLR11 to recognise flagellin, a TLR5 ligand, chicken TLR5 is a candidate receptor for *Eimeria* profilin. The SEAP reporter gene assay is under development for TLR1LA in combination

with TLR2A and B, and TLRs 4 and 5, but due to high background readings in mock-transfected HEK293T-SEAP cells and failure of positive controls to induce SEAP expression it was not possible to test these TLRs in this study.

In conclusion, BMM and BMDC produce proinflammatory cytokines in response to EtAg. BMM had a more regulatory phenotype characterised by the expression of *IL10* in response to EtAg whereas BMDC were more Th1-promoting in their expression of *IL12A* and *IFNG*. We show that potential vaccine candidates IMP and AMA1 can be recognised via TLR1LB/2A heterodimers and that BMM responded to these antigens by upregulating the expression of *NOS2* and *IL6*. During *Eimeria* infection, macrophages are able to recognise IMP1 and AMA1 and orchestrate both the innate inflammatory response and the adaptive response in order to facilitate protective immunity in response to vaccination with both IMP1 and AMA1. Purification of recombinant IMP1 and AMA1 would allow for more robust testing of BMM responses to these vaccine candidates and further characterisation of the ability of BMM to produce Th-promoting cytokines could be explored. Further development of the SEAP assay for TLR1LA/2A and TLR1LA/2B heterodimers, TLR4 and TLR5 would present an exciting opportunity to determine if these PRR can recognise *Eimeria* antigens, in particular GPI-anchored SAGs (by TLR1LA/B and TLR2A/B heterodimers) and *Eimeria* profilin (by TLR5).

Chapter 4 Immune response of Ross 308 broilers to infection with *E. maxima* and *E. tenella*

4.1 Introduction

The fact that *Eimeria* induces a predominantly IFNγ/Th1-mediated immune response is well established (Rose *et al.*, 1979; Rose *et al.*, 1989; Rose *et al.*, 1991; Yun *et al.*, 2000; Kwa *et al.*, 2006) however the responses of Th17 cells during *Eimeria* infection are not well characterised. Infection with *Eimeria* induces an inflammatory response that can include many of the driving forces behind a Th17 response; increases to IL-1β (Laurent *et al.*, 2001), IL-6 (Lynagh *et al.*, 2000) and TGF-β4 (Jakowlew *et al.*, 1997) have been reported; providing the ideal context for induction of a Th17-mediated response. In mammals, naïve T cells are driven towards a Th17 phenotype by antigen presentation to naïve $CD4^+$ cells via MHCII in the presence of TGF-β4, IL-1β, IL-6, IL-21 and IL-23 cytokines. IL-17A, IL-17F, IL-21 and IL-22 are considered Th17 effector cytokines. To date, studies attempting to elucidate Th17 responses during *Eimeria* infection have shown IL-17A mRNA to be up-regulated in duodenal IELs during *E. acervulina* infection and jejunal IELs during *E. maxima* infection (Hong *et al.*, 2006a; Hong *et al.*, 2006b) but unchanged in caecal IELs during *E. tenella* infection (Hong *et al.*, 2006a). Increased IL-17F mRNA was also observed in the gut following *E. maxima* infection (Kim *et al.*, 2014). Th17 cytokines have also been implicated in immunopathology during *E. tenella* infection in chickens where IL-17A was neutralised with an antibody, resulting in decreased oocyst shedding, reduced lesion score and increased weight gains (Zhang *et al.*, 2013). During *E. falciformis* infection, antibody-mediated neutralisation of IL-17A and IL-22 increased parasite burden in IFNγ knockout mice however reduced weight loss was also observed (Stange *et al.*, 2012).

The hypothesis is that Th17 cells are involved in the immune response to *Eimeria,* and that the Th17 response contributes to inflammation-induced pathology and is important to limiting parasite replication in the gut during *E. maxima* and *E. tenella* infection. This chapter aims to identify a Th17 response in the intestinal tract by measuring the expression of Th17-associated cytokines by RT-qPCR analysis during *Eimeria* infection in Ross 308 broilers that have been given a primary inoculation of either *E. maxima* or *E. tenella*. To characterise Th17 responses, gene expression of Th17 driver and effector cytokines *TGFB4*, *IL17A*, *IL17F*, *IL21*, *IL22* and *IL23R* was measured. Target genes for RT-qPCR also included *IFNG* and *IL13*. In both mammals and chickens, IFNγ is a proinflammatory cytokine and is considered the main driving and effector force behind a Th1 response (Guo *et al.*, 2013). IL-13 is considered a Th2 effector cytokine, but can also be produced by other cell types including macrophages and can suppress inflammatory activities of monocytes, enhance B cell proliferation and promote Ig isotype switching (Powell *et al.*, 2009). Increased IL-10 has previously been implicated in susceptibility to *E. maxima* infection in line 15I White Leghorn chickens (Rothwell *et al.*, 2004) and was also studied.

In addition, changes to the cell populations within the jejunum following *E. maxima*, and the caecum following *E. tenella* infection were evaluated by ICC. To analyse changes to different T cell populations within the gut, antibodies to various T cell markers including CD4, CD8α, TCRαβ$_1$, TCRαβ$_2$, TCRγδ and CD25 were utilised. CD25 is present on activated T cells (Hála *et al.*, 1986) and NK cells (Neulen *et al.*, 2015).

The Bu-1 antibody recognises chB6 present on all B cells but not plasma cells (Houssaint *et al.*, 1987; Rothwell *et al.*, 1996; Tregaskes *et al.*, 1996) and was used to study the B cell population during *Eimeria* infection.

Currently, there is no single defining marker for NK cells available. In the chicken, NK cells are abundant in the intestinal epithelium. Chicken NK cells can express CD8αα^{+} (Göbel *et al.*, 2001) but, upon activation, will down-regulate expression of CD8α (Jansen *et al.*, 2010) and up-regulate expression of CD25^{+} (Neulen *et al.*, 2015). A subset of NK cells that express chB6 are also present in the intestinal epithelium (Vervelde *et al.*, 1993a).

To assess APC populations during the course of *Eimeria* infection two antibodies were used. The first, KUL01, recognises chicken mannose receptor 1 (MRC1L-B) present on macrophages (Staines *et al.*, 2014). The second, an antibody to the chicken T cell immunoglobulin mucin domain containing protein (Tim4; clone JH9) that is present on APC subpopulations and TCRγδ^{+} cells recognises phosphatidylserine on apoptotic cells. As in mammals, chicken Tim4 on APC is the ligand for Tim1 on T cells and their interaction regulates T cell proliferation. The JH9 antibody recognises both the short and long isoforms of Tim4 (Hu *et al.*, 2016).

Combining RT-qPCR gene expression data and ICC analysis of cell populations within the gut during infection will provide insight into the cellular mechanisms underpinning immune responses to *Eimeria* infection and provide a basis for further study into which cell types are responsible for the production of which cytokines during infection.

4.2 Results

4.2.1 Body weight gain after infection with *E. maxima* and *E. tenella*

The weight of each bird was recorded at 2 dpi and then again before they were culled to calculate body weight gain during infection. Infection with *E. maxima* and *E. tenella* did not affect weight gain until day 15, when *E. maxima*-infected birds had significantly ($p<0.05$) lower weight gain than control birds (Figure 4-1).

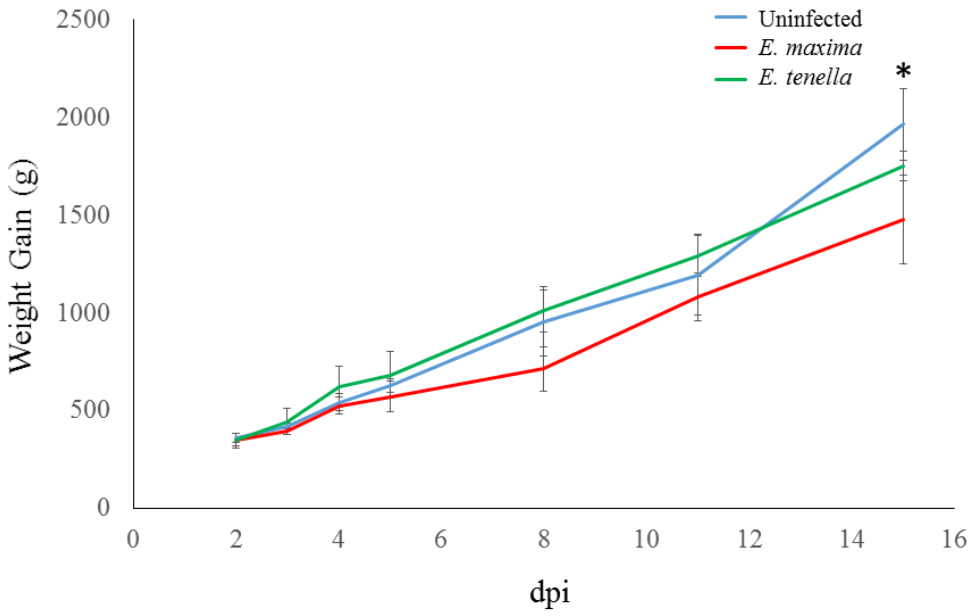

Figure 4-1: Weight gains (g) in Ross 308 broilers infected with either *E. maxima* or *E. tenella* as compared to uninfected birds. Three-week-old Ross 308 broilers were orally inoculated with 2 x 10^3 *E. maxima* or *E. tenella* oocysts. Weight gains were calculated in individual birds from 2 dpi to time of culling. Shown are the mean weight gains ± SD for each time point; n=3 for control and n=5 for infected groups. An asterisk denotes a significant difference ($p<0.05$; Mann-Whitney U test) in the weight of *E. maxima*-infected broilers as compared to uninfected control birds at 15 dpi.

4.2.2 *E. maxima* and *E. tenella* replication in the gut

As a measure of parasite replication, qPCR was employed to measure the quantity of parasite genomic DNA in the jejunum and caecum during the course of *E. maxima* and *E. tenella* infection. No parasite DNA was detected in any of the control birds. *E. maxima* genomic DNA was first detected in the jejunum from 4 to 8 dpi and peaked 5 dpi (Figure 4-2A). *E. tenella* DNA was first detected in the caeca at 3 dpi, peaked at 6 dpi and was not cleared completely during the course of the experiment (Figure 4-2B).

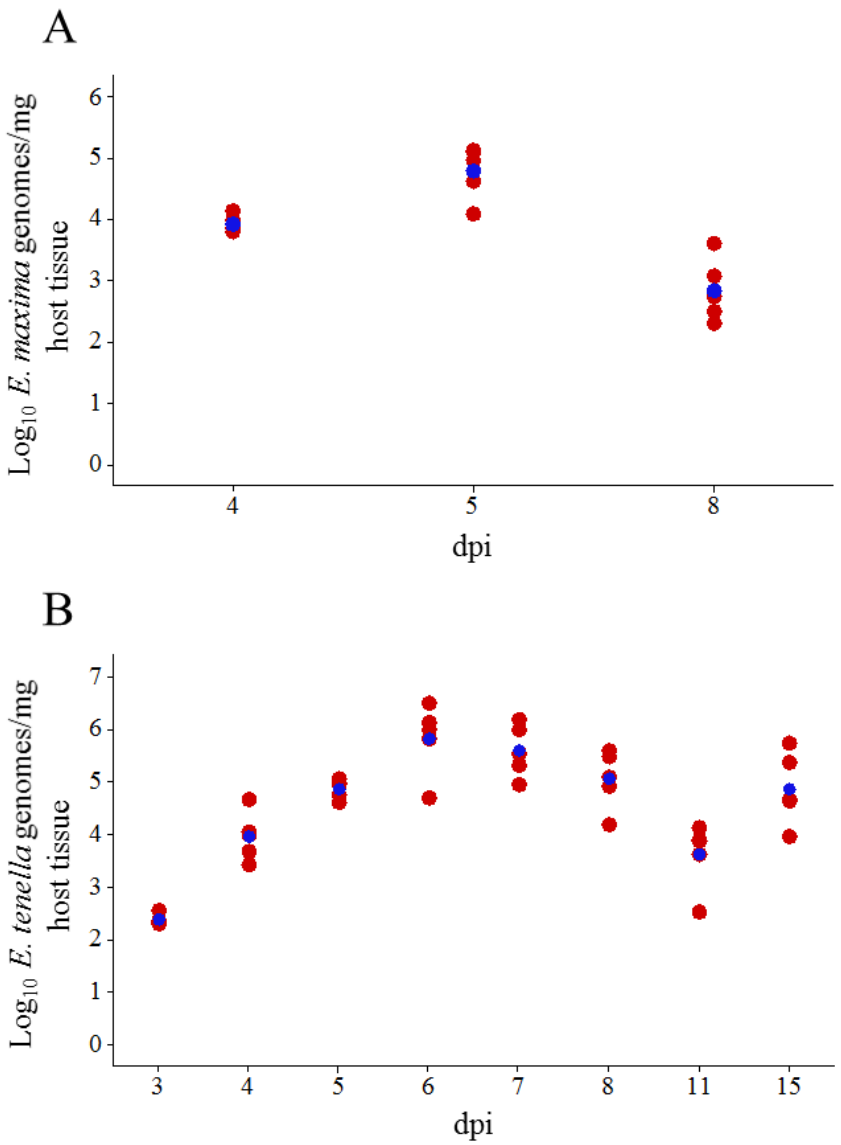

Figure 4-2: Parasite replication in the jejunum of *E. maxima*-infected and caecum of *E. tenella*-infected Ross 308 broilers as determined by qPCR. *E. maxima* (A) was quantified by an established qPCR method targeting the MIC1 gene (Blake *et al*., 2006). *E. tenella* (B) was quantified by a qPCR reaction targeting the RAPD-SCAR marker Tn-E03-1161 (Nolan *et al*., 2015). For *E. maxima*-infected and control jejunum samples, 10 cm of jejunum spanning 5 cm either side of the MD was collected for genomic DNA extraction. For *E. tenella*-infected samples and control birds, both of the remaining caeca were pooled for genomic DNA extraction. All parasite quantification was performed by Dr Matt Nolan (RVC, UK). Shown are the $\log_{10}$ genomes per mg of host tissue. Red circles represent values from individual birds and blue circles represent the mean of 5 birds.

4.2.3 Changes to cytokine expression in the gut after *E. maxima* and *E. tenella* infection

RNA was extracted from the mid-caecum and jejunum of *E. tenella*- and *E. maxima*-infected birds respectively and changes to the mRNA levels of cytokines were quantified by TaqMan® RT-qPCR as in section 2.2. To check whether a particular gene was altered during infection, RNA samples were first pooled within each group at each time point before RT-qPCR analysis to screen for changes in gene expression. Two µg of total RNA from each sample at 2, 4, 8 and 15 dpi was pooled and total volumes made up to 20 µl with RNase-free water. If the expression of genes changed following infection expression was measured in individual birds. No changes were seen in the expression of *TGFB4*, *IL23R* (Figure 4-3) *IL17A* or *IL17F* (Figure 4-4) mRNA from RT-qPCR analysis of the pooled RNA sample set. No changes were observed in *IL22* mRNA following infection with either *Eimeria* species except for *E. maxima* infected jejunum at 15 dpi, where *IL22* was not detectable in the control sample (Figure 4-4). The mRNA of this sample was of satisfactory quality when measured using a Spectrophotometer, the RNA concentration was 611.9 ng/ml, the 260/280 ratio was 2.05 and the 260/230 ratio was 2.19. The RT-qPCR reaction was repeated for individual samples from 15 dpi and was detectable in all control birds at 15 dpi at similar levels to that of infected birds indicating that there was no change in IL-22 mRNA at this time point. The reason why IL-22 mRNA was not detectable in the pooled sample set for control birds at 15 dpi is unclear.

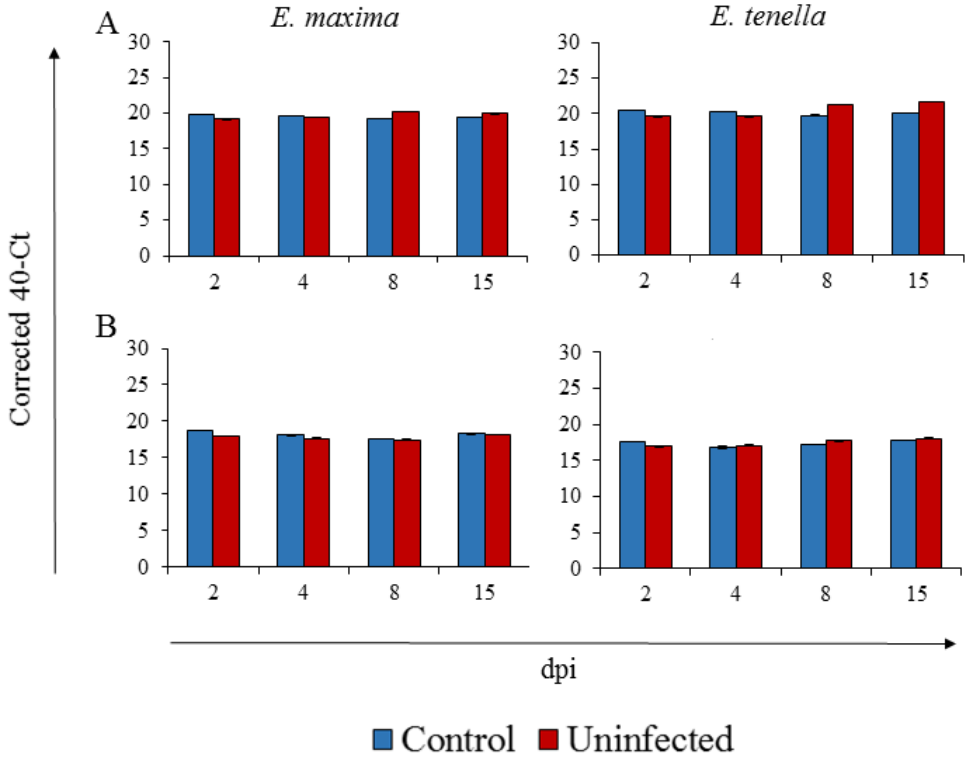

Figure 4-3: Expression of Th17 driving genes *TGFB4* (A) and *IL23R* (B) mRNA in the jejunum of *E. maxima*-infected and mid-caecum of *E. tenella*-infected Ross 308 broilers as quantified by TaqMan® RT-qPCR. Three-week-old broilers were orally inoculated with 2 x 10^3 oocysts of either *E. tenella* or *E. maxima* and tissues collected at various time points post infection. RNA samples were pooled within groups (n=3 for control and n=5 for infected birds). Data presented are the mean corrected 40-Ct values for triplicate wells ± SEM.

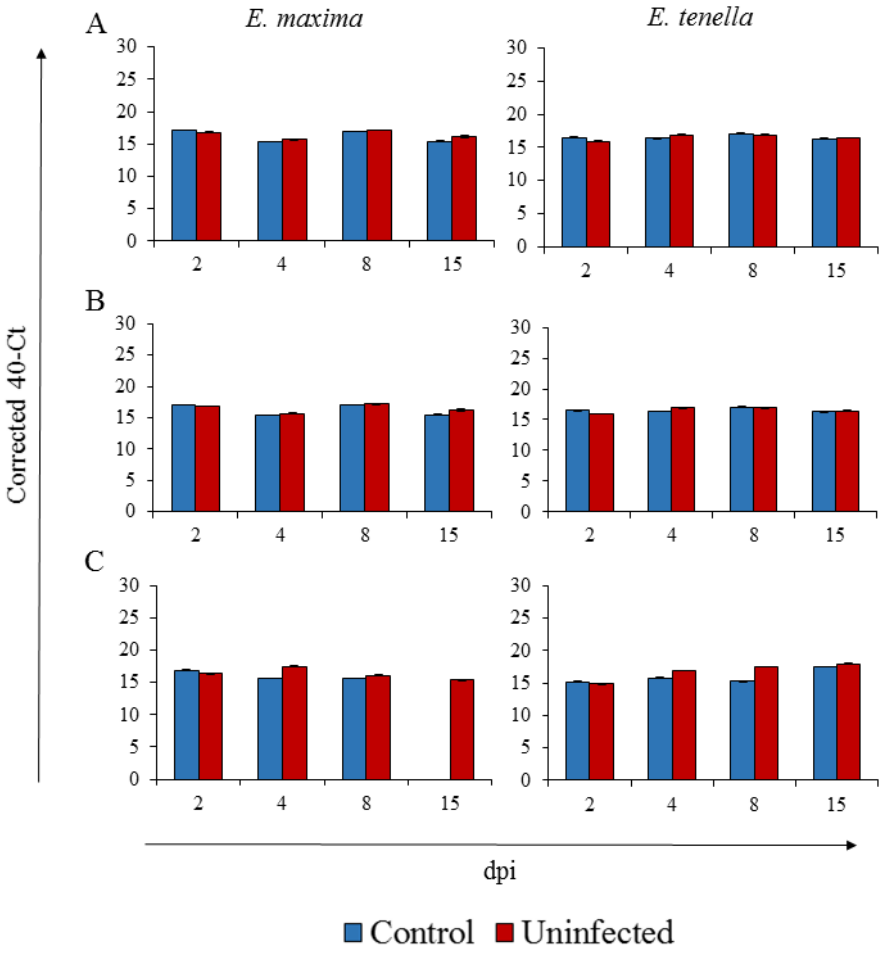

Figure 4-4: Expression of Th17 effector cytokines *IL17A* (A), *IL17F* (B) and *IL22* (C) mRNA expression in the jejunum of *E. maxima*-infected and mid-caecum of *E. tenella*-infected Ross 308 broilers as quantified by TaqMan® RT-qPCR. Three-week-old broilers were orally inoculated with 2 x 10^3 oocysts of either *E. tenella* or *E. maxima* and tissues collected at various time points post infection. RNA samples were pooled within groups (n=3 for control and n=5 for infected birds). Data presented are the mean corrected 40-Ct values for triplicate wells ± SEM.

From initial screening of pooled RNA samples, *IL21* mRNA was the only Th17-associated cytokine up-regulated following *Eimeria* infection and therefore the expression of *IL21* mRNA was measured in individual birds (Figure 4-5). *IL21* mRNA levels were increased significantly ($p<0.05$) from 4 dpi following *E. maxima* infection and 5 dpi (although not at 11 dpi) following *E. tenella* infection.

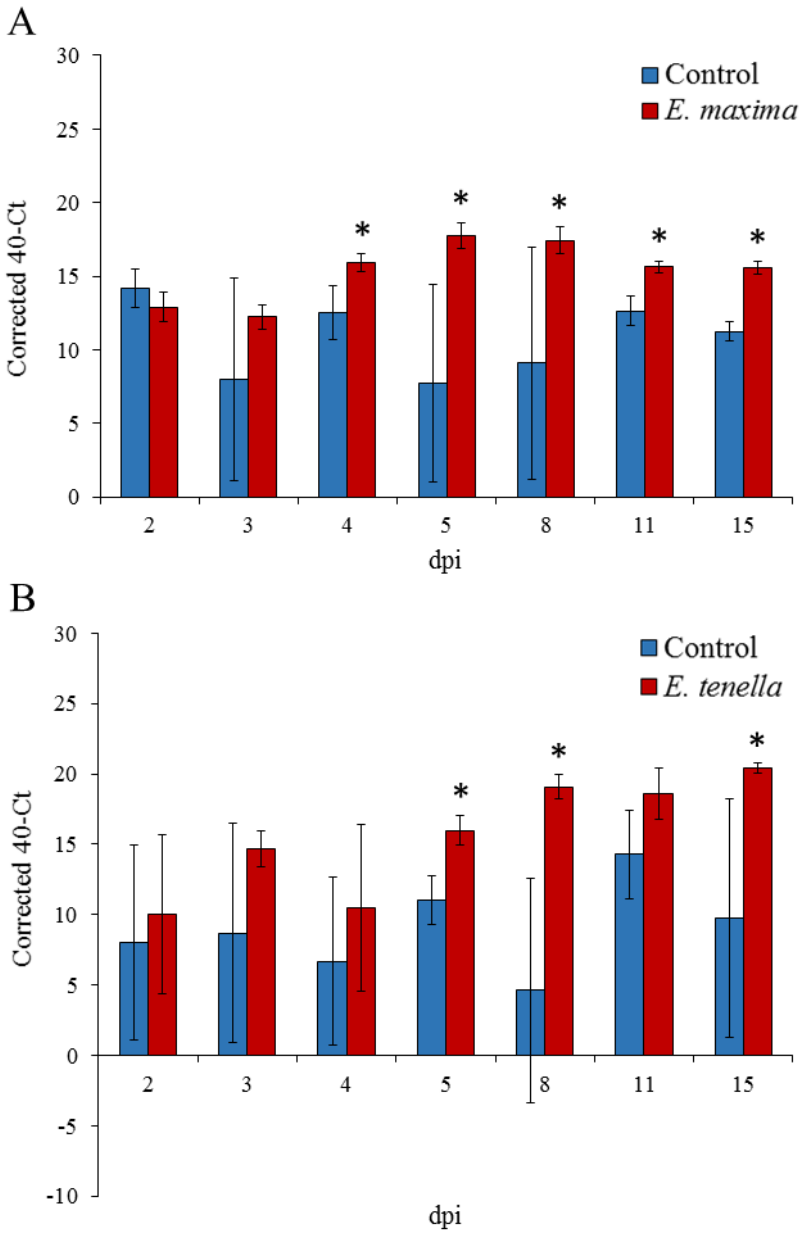

Figure 4-5: *IL21* mRNA expression in the jejunum of *E. maxima*-infected (A) and mid-caecum of *E. tenella*-infected (B) Ross 308 broilers as quantified by TaqMan® RT-qPCR. Three-week-old broilers were orally inoculated with 2 x10^3 oocysts of either *E. tenella* or *E. maxima* and tissues collected at various time points post infection. Data are presented as the mean corrected 40-Ct values for each group ± SD. Asterisks indicate significant differences between infected groups (n=5) compared to controls (n=3; $p<0.05$, Mann-Whitney U Test).

IL-15 has been shown to work in synergy with IL-21 to enhance vaccine efficacy in *T. gondii* infection in mice (Li *et al.*, 2014) and *IL15* mRNA was therefore measured in the pooled RNA sample set. There was no change to *IL15* mRNA expression following either *E. maxima* or *E. tenella* infection (Figure 4-6).

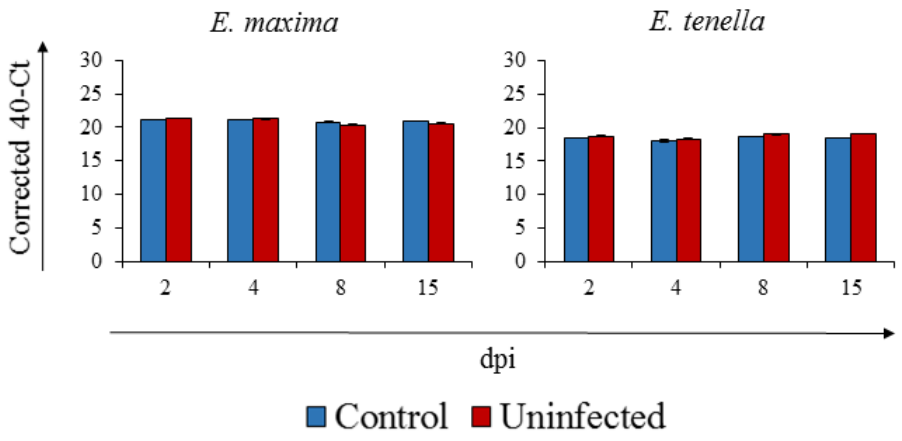

Figure 4-6: *IL15* mRNA expression in the jejunum of *E. maxima*-infected and mid-caecum of *E. tenella*-infected Ross 308 broilers as quantified by TaqMan® RT-qPCR. Three-week-old broilers were orally inoculated with 2 x 10^3 oocysts of either *E. tenella* or *E. maxima* and tissues collected at various time points post infection. RNA samples were pooled within groups (n=3 for control and n=5 for infected birds). Data presented are the mean corrected 40-Ct values for triplicate wells ± SEM.

To check for a Th1 response, expression of *IFNG* and *IL2* mRNA was measured. During *E. maxima* infection, *IFNG* mRNA was increased significantly ($p<0.05$) in the jejunum from 4 dpi onwards and from 5 dpi onwards in the caecum during *E. tenella* infection when compared to controls (Figure 4-7). *IL2* mRNA was significantly ($p<0.05$) up-regulated following *E. maxima* and *E. tenella* infection (Figure 4-8).

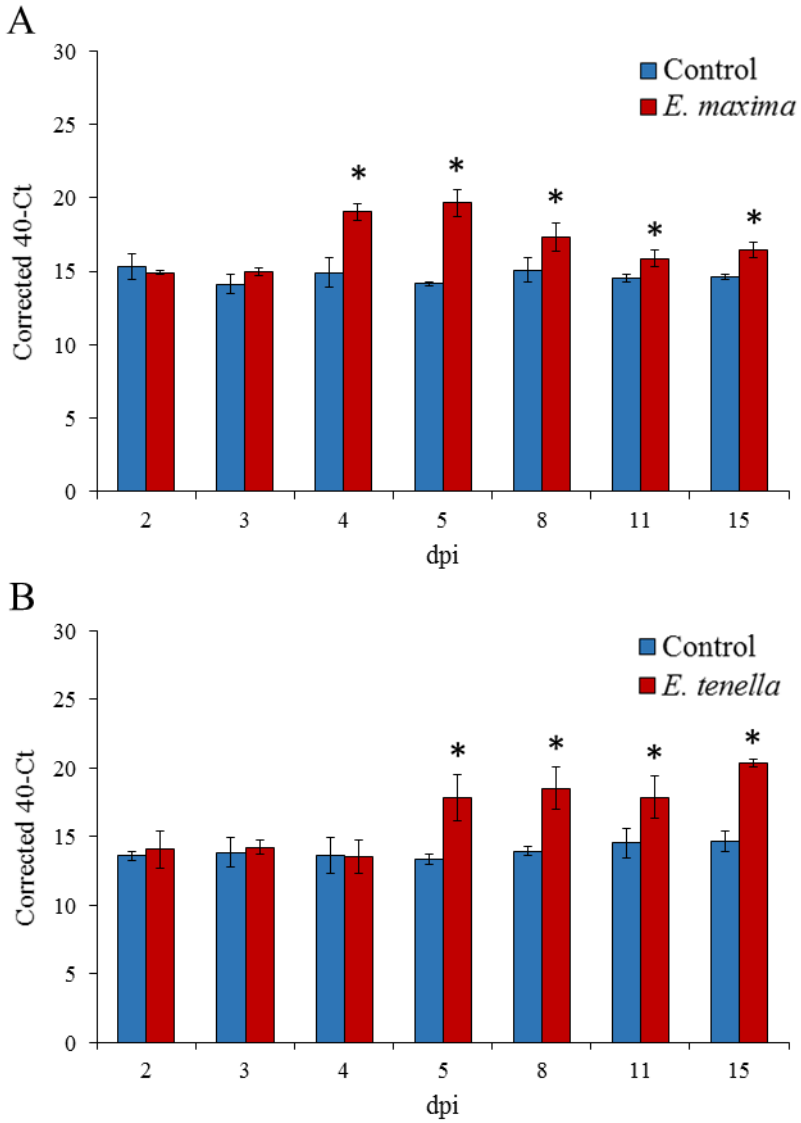

Figure 4-7: *IFNG* mRNA expression in the jejunum of *E. maxima*-infected (A) and mid-caecum of *E. tenella*-infected (B) Ross 308 broilers as quantified by TaqMan® RT-qPCR. Three-week-old broilers were orally inoculated with 2 x 10^3 oocysts of either *E. tenella* or *E. maxima* and tissues collected at various time points post infection. Data are presented as the mean corrected 40-Ct values for each group ± SD. Asterisks indicate significant differences between infected groups (n=5) compared to controls (n=3; $p<0.05$, Mann-Whitney U Test).

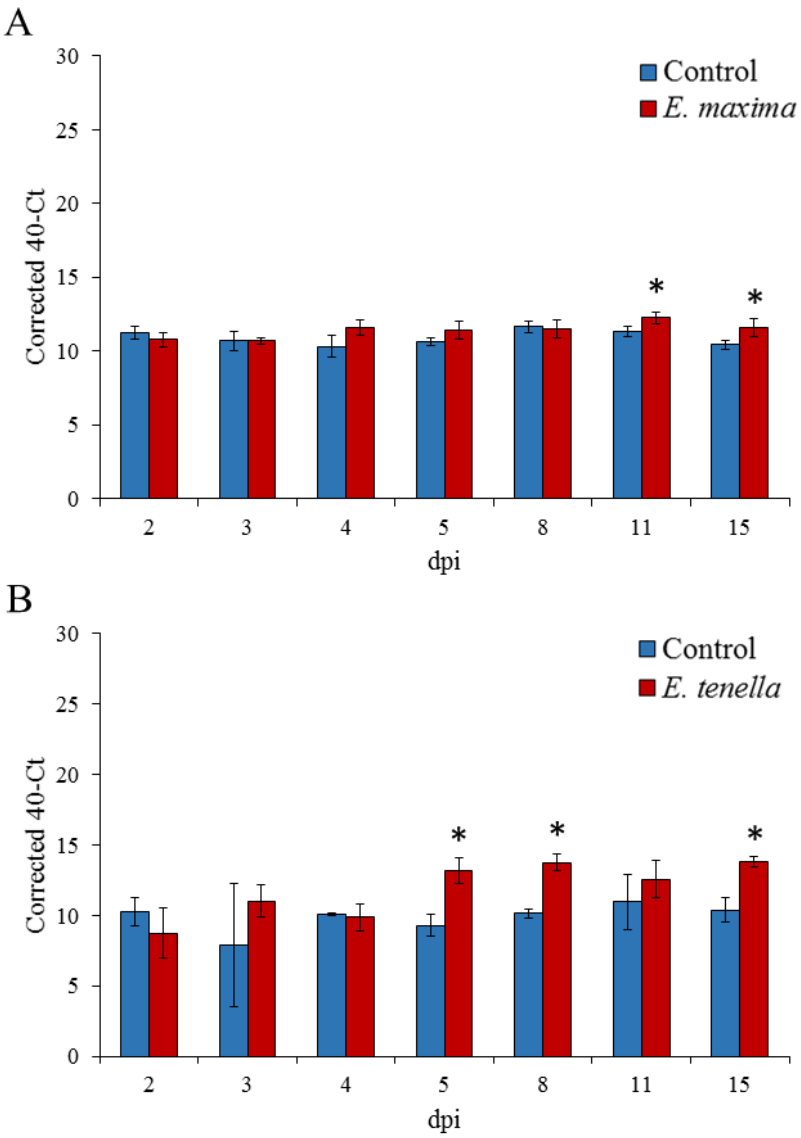

Figure 4-8: *IL2* mRNA expression in the jejunum of *E. maxima*-infected (A) and mid-caecum of *E. tenella*-infected (B) Ross 308 broilers as quantified by TaqMan® RT-qPCR. Three-week-old broilers were orally inoculated with 2 x 10^3 oocysts of either *E. tenella* or *E. maxima* and tissues collected at various time points post infection. Data are presented as the mean corrected 40-Ct values for each group ± SD. Asterisks indicate significant differences between infected groups (n=5) compared to controls (n=3; $p<0.05$, Mann-Whitney U Test).

To confirm the absence of a Th2 response, *IL13* mRNA was measured in the pooled RNA sample set. As expected, no changes were seen in the expression of *IL13* mRNA compared with control bird tissues (Figure 4-9) during analysis of the pooled RNA sample set therefore RT-qPCR analysis on individual birds was not repeated on these samples.

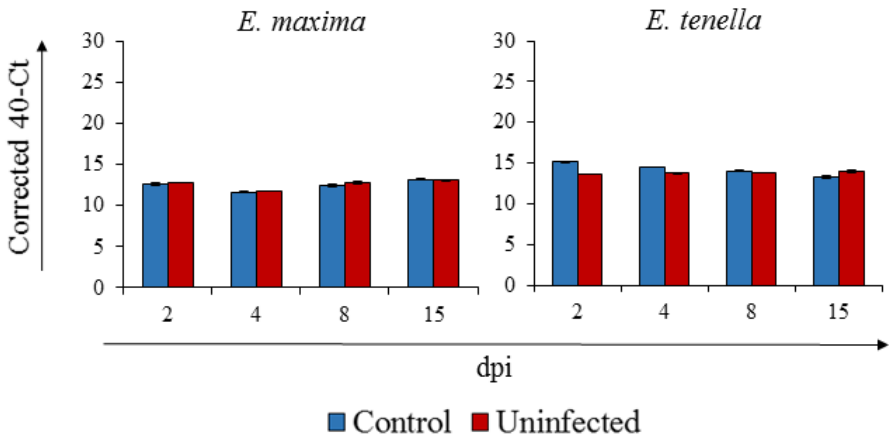

Figure 4-9: *IL13* mRNA expression in the jejunum of *E. maxima*-infected and mid-caecum of *E. tenella*-infected Ross 308 broilers as quantified by TaqMan® RT-qPCR. Three-week-old broilers were orally inoculated with 2 x10^3 oocysts of either *E. tenella* or *E. maxima* and tissues collected at various time points post infection. RNA samples were pooled within groups (n=3 for control and n=5 for infected birds). Data presented are the mean corrected 40-Ct values for triplicate wells ± SEM.

In this chapter, the primers and probe set used to quantify *IL10* mRNA was IL10 (2) as given in Table 2-1 (Chapter 2). *IL10* mRNA was up-regulated significantly ($p<0.05$) in the jejunum and caecum of *E. maxima* and *E. tenella* infected birds respectively (Figure 4-10).

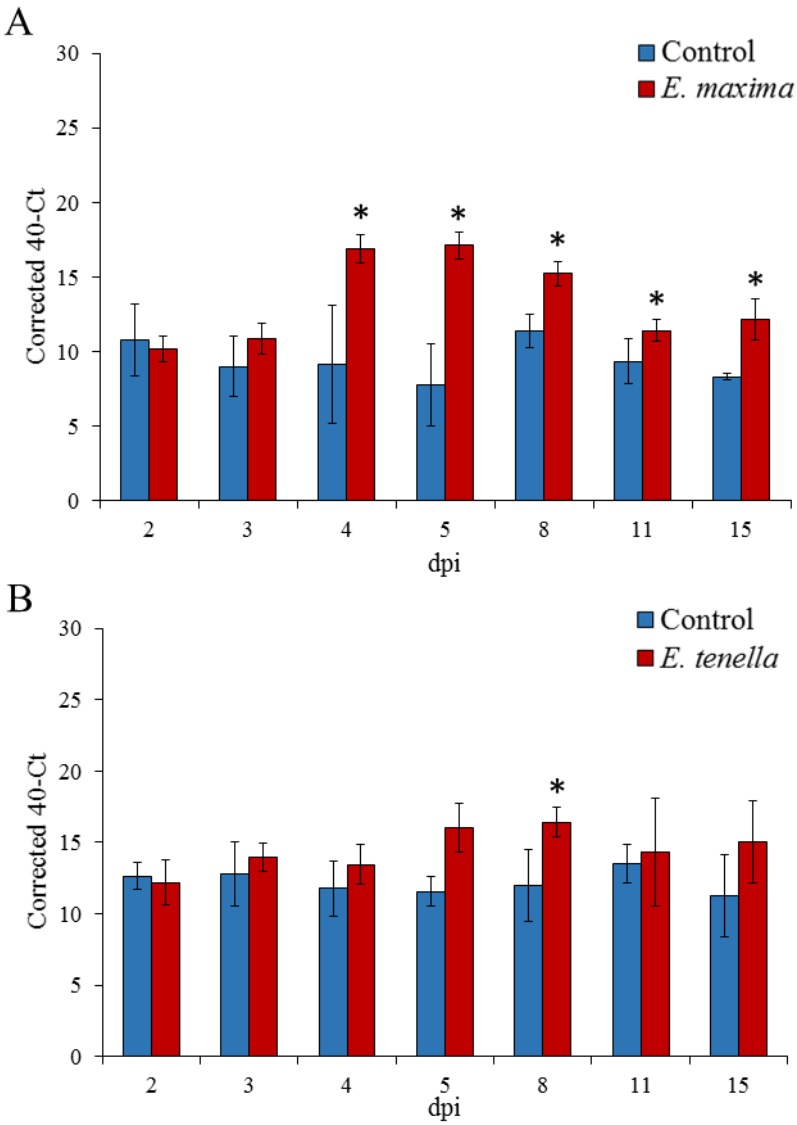

Figure 4-10: *IL10* mRNA expression in the jejunum of *E. maxima*-infected (A) and mid-caecum of *E. tenella*-infected (B) Ross 308 broilers as quantified by TaqMan® RT-qPCR. Three-week-old broilers were orally inoculated with 2 x 10^3 oocysts of either *E. tenella* or *E. maxima* and tissues collected at various time points post infection. Data are presented as the mean corrected 40-Ct values for each group ± SD. Asterisks indicate significant differences between infected groups (n=5) compared to controls (n=3; $p<0.05$, Mann-Whitney U Test).

As the levels of *IFNG*, *IL21*, *IL2* and *IL10* mRNA were changed following *E. maxima* and *E. tenella* infection, the relationship between the level of *IFNG*, *IL21*, *IL2* and *IL10* expression and *Eimeria* parasite burden in the gut during infection was examined (Table 4-1).

Table 4-1: Spearman's rank correlation coefficients between IFNG, IL21, IL2 and IL10 mRNA expression and *Eimeria* parasite copy number in the jejunum following *E. maxima* infection and mid-caecum following *E. tenella* infection. Correlation coefficients were calculated between mean corrected 40-Ct values of triplicate wells for cytokines as measured in individual birds and the parasite genome copy number per mg of host tissue. Shown is the Spearman's correlation coefficient for data of all infected birds pooled from all time points (n = 35). A coefficient value between -1 and 0 indicates a negative correlation, a coefficient value of 0 indicates no correlation and a coefficient value between 0 and 1 indicates a positive correlation. P values were calculated using the two-tailed t test and were considered significant at $p<0.05$ and highly significant at $p<0.001$.

cytokine	*E. maxima*	*E. tenella*
IFN-γ	0.85 ($p<0.001$)	0.68 ($p<0.001$)
IL-21	0.72 ($p<0.001$)	0.59 ($p<0.001$)
IL-2	0.45 ($p<0.05$)	-0.11 ($p=0.51$)
IL-10	0.86 ($p<0.001$)	0.63 ($p<0.001$)

During *E. maxima* infection, higher levels of *IFNG*, *IL21*, *IL2* and *IL10* mRNA in the jejunum correlated positively with higher *E. maxima* copy number in infected chickens. Higher levels of *IFNG*, *IL21* and in particular *IL10* mRNA, were strongly correlated with the quantity of parasite detected and were all highly significant at $p<0.001$. Increased *IL2* mRNA was only weakly correlated with higher *E. maxima* copy number and was not significant.

4.2.4 Changes to cell populations in the gut after *E. maxima* and *E. tenella* infection

To assess changes to the subpopulation of immune cells in the gut during *Eimeria* infection, ICC analysis was performed on snap frozen tissues. Tissues snap-frozen at collection for ICC from the commercial bird trial were stored at -80°C. The tissues suffered severe freezer burn during storage and as a result, tissues would not adhere sufficiently to slides to withstand the staining protocol. To rehydrate the tissues, they were removed from the filter paper and re-embedded in OCT medium by the following method. Cryostat moulds were filled with OCT medium into which tissues were placed and oriented appropriately. The tissues were then promptly frozen on an aluminium boat over liquid nitrogen before being stored at -80°C wrapped in foil and in zip lock bags until required for sectioning.

Due to the nature of the ICC stains whereby the main changes to cell populations were highly localised to where the *Eimeria* is located within the gut, it was decided that the optimum way to determine changes to cell populations was by way of a scoring system to descriptively quantify changes as detailed in section 2.6.2 (Chapter 2). The results are shown here alongside histology images of the gut. Images of a

higher magnification are also shown where an increase in the IEL subpopulation was observed.

In jejunum from control birds, the vast majority of $CD4^+$ cells were LPLs, evenly spaced throughout the lamina propria of the crypts and villi and were round in morphology. The presence of $CD4^+$ IELs was rare in the jejunum. The number of $CD4^+$ LPLs increased from 5 dpi following *E. maxima* infection. Clusters of $CD4^+$ LPLs were observed in the crypts of the jejunum in two out of four of the *E. maxima*-infected birds at 5 dpi and in all birds at 8 dpi. At 15 dpi, in only one bird, clusters of LPLs expressing CD4 were observed in the jejunum following *E. maxima* infection. Overall, there was no change in $CD4^+$ IELs in the jejunum following *E. maxima* infection (Figure 4-11 and Figure 4-12).

In control caeca, all $CD4^+$ cells were LPLs as no $CD4^+$ IELs were observed. $CD4^+$ LPLs were evenly scattered throughout the lamina propria of the crypts and villi and were round in shape in controls. The number of $CD4^+$ LPLs increased from 3 dpi during *E. tenella* infection. At 3 dpi in one bird from the *E. tenella*-infected group, clusters of $CD4^+$ LPLs were observed and from 5 dpi onwards, clusters of $CD4^+$ LPLs were observed in almost all the mid-caeca crypts of *E. tenella*-infected birds. At no point were $CD4^+$ IELs observed in the caecum of *E. tenella*-infected birds (Figure 4-11 and Figure 4-13).

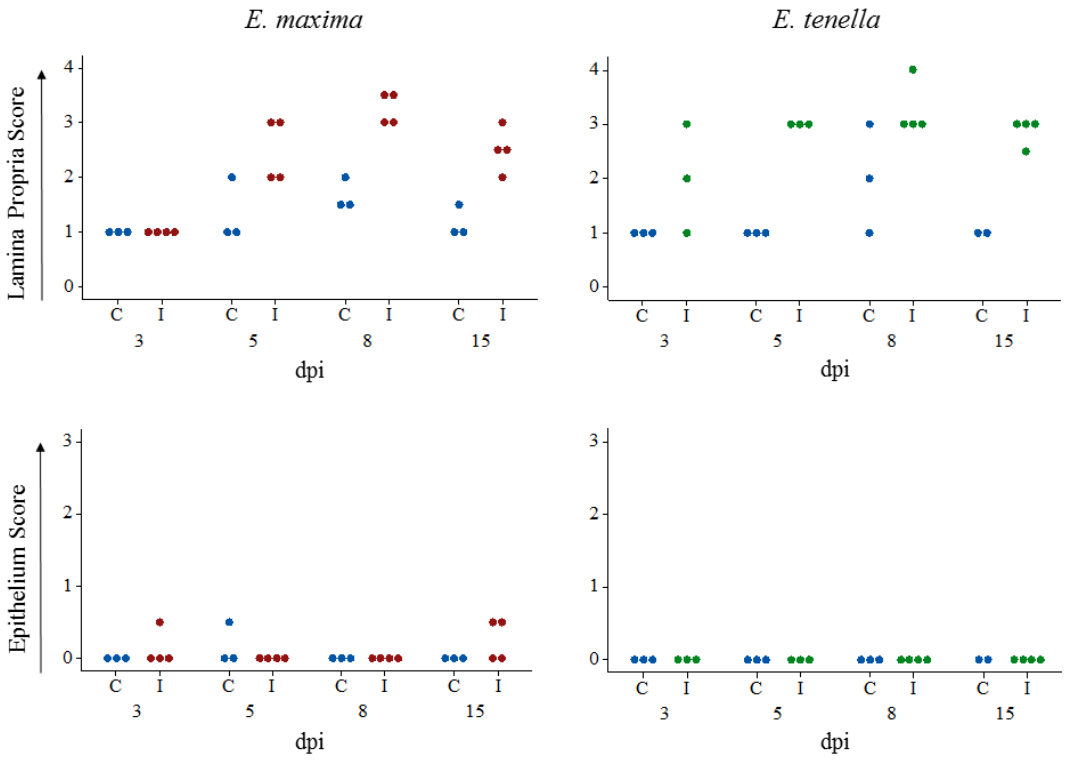

Figure 4-11: CD4^{+} cells in the jejunum of *E. maxima*-infected and mid-caecum of *E. tenella*-infected Ross 308 broilers. Three-week-old Ross 308 broilers were orally inoculated with 2 x 10^{3} oocysts of either *E. maxima* or *E. tenella* and samples collected for histology at 3, 5, 8 and 15 dpi. Cell populations were scored as in section 2.6.2. Control (C) tissues are shown in blue and infected (I) tissues are shown in red for *E. maxima* and green for *E. tenella* infection. Each individual dot represents data from one bird.

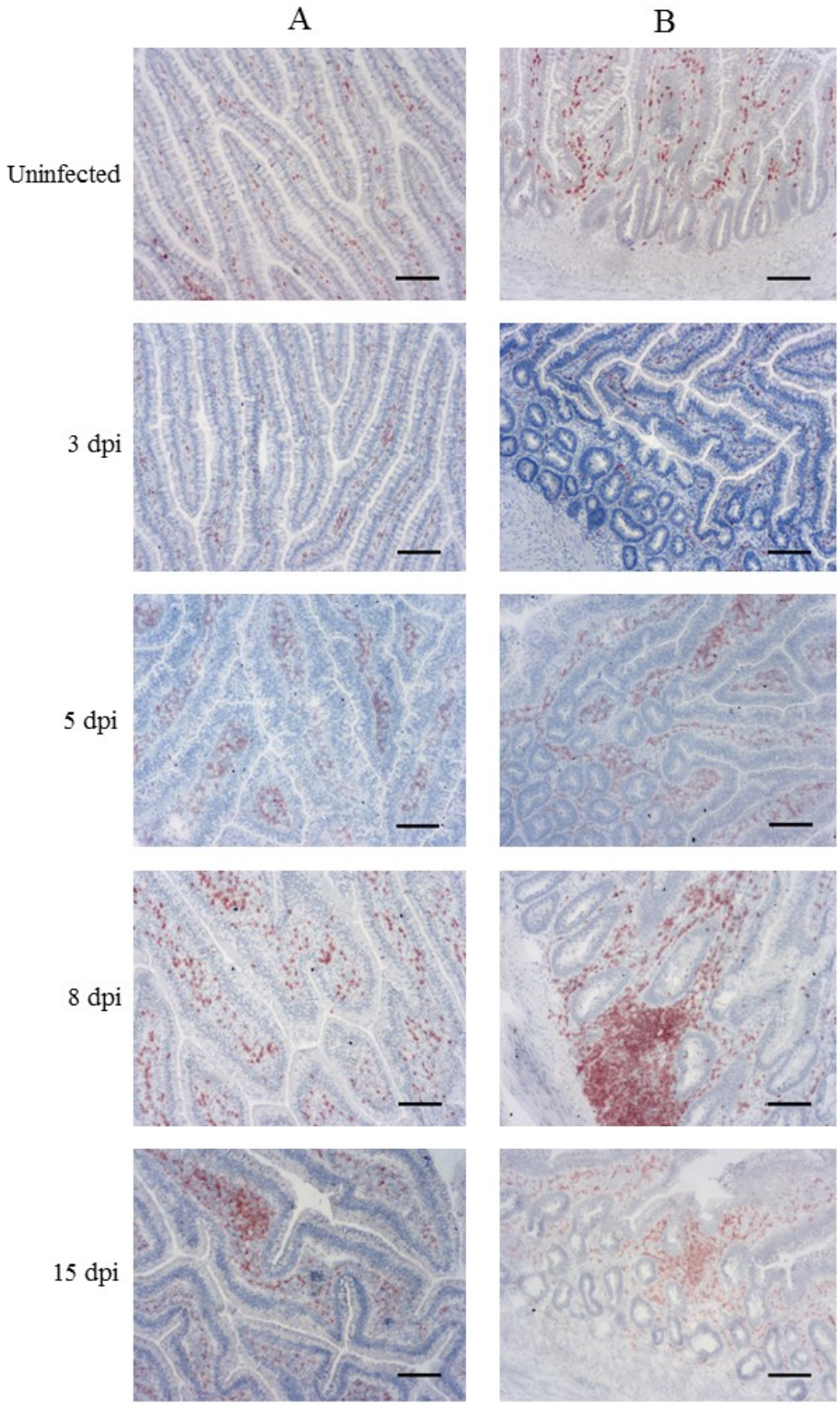

Figure 4-12: $CD4^+$ cells in the villi (A) and crypts (B) in the jejunum of *E. maxima*-infected Ross 308 broilers. Three-week-old birds were orally inoculated with 2 x 10^3 *E. maxima* oocysts and samples collected for histology at 3, 5, 8 and 15 dpi. Red AEC stain indicates $CD4^+$ cells. Bars represent 100 µm.

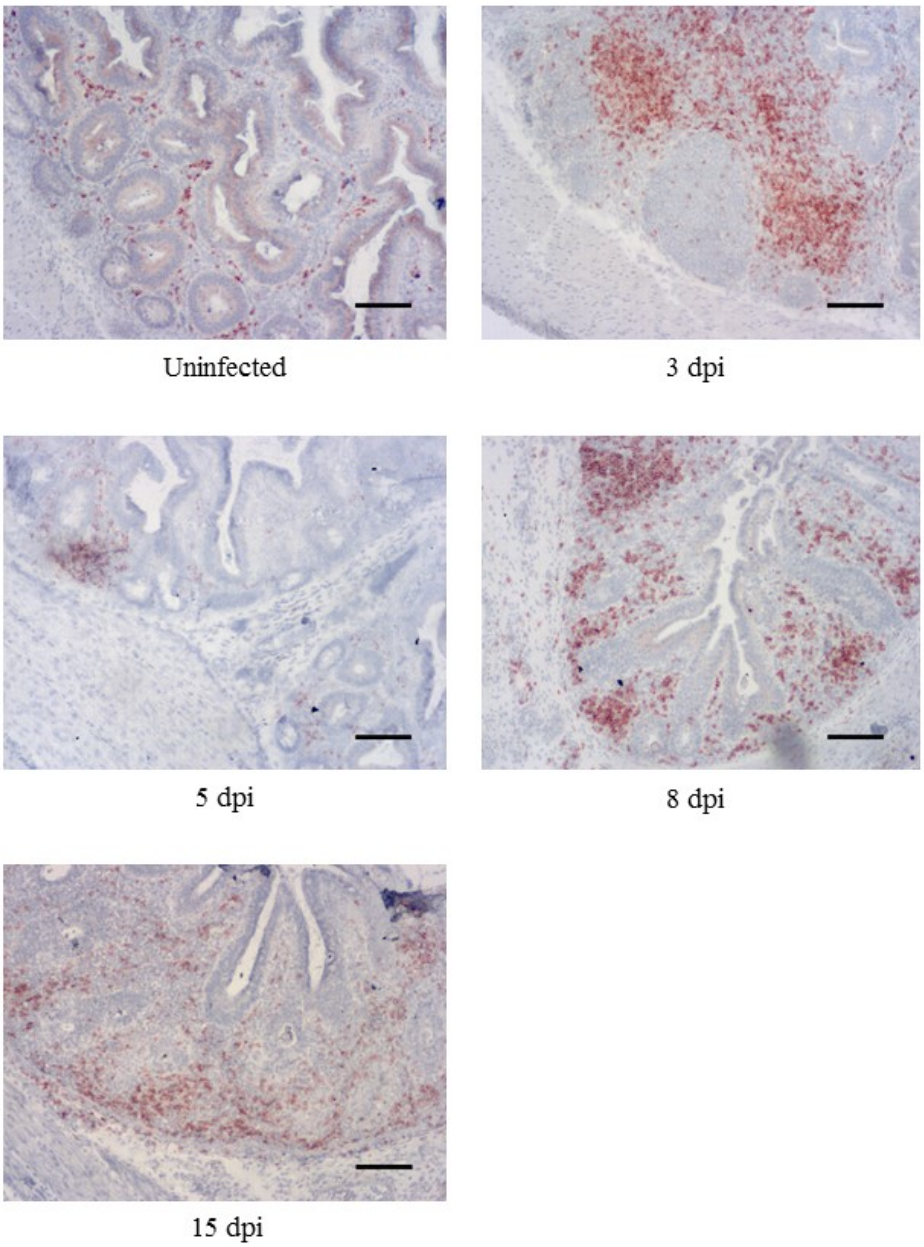

Figure 4-13: $CD4^+$ cells in the mid-caecum of *E. tenella*-infected Ross 308 broilers. Three-week-old birds were orally inoculated with 2 x 10^3 *E. tenella* oocysts and samples collected for histology at 3, 5, 8 and 15 dpi. Red AEC stain indicates $CD4^+$ cells. Bars represent 100 µm.

In control jejunum, CD8α$^+$ cells were located in both the lamina propria and the epithelium. CD8α$^+$ LPLs were rounded in shape and scattered throughout the lamina propria. Many of the CD8α$^+$ IELs present were elongated in shape and pressed against the basal membrane and some were rounder cells positioned throughout the epithelium. The majority of CD8α$^+$ cells in control jejunum were observed throughout the villi and in the areas of the crypts closer to the bases of the villi. Following *E. maxima* infection, an increase in CD8α$^+$ LPLs was observed from 5 dpi onwards in all infected birds compared to control baseline levels. In three out of four *E. maxima*-infected birds, clusters of CD8α$^+$ LPLs were observed in the jejunum crypts at 5 dpi and in two of four birds at 8 dpi. By 15 dpi, no clusters were observed in the crypts but the numbers of CD8α$^+$ LPLs were still elevated compared to the control tissues (Figure 4-14 and Figure 4-15). Increases were also seen in the number of CD8α$^+$ IELs present in the epithelium at 8 and 15 dpi with *E. maxima* (Figure 4-14, Figure 4-15 and Figure 4-16).

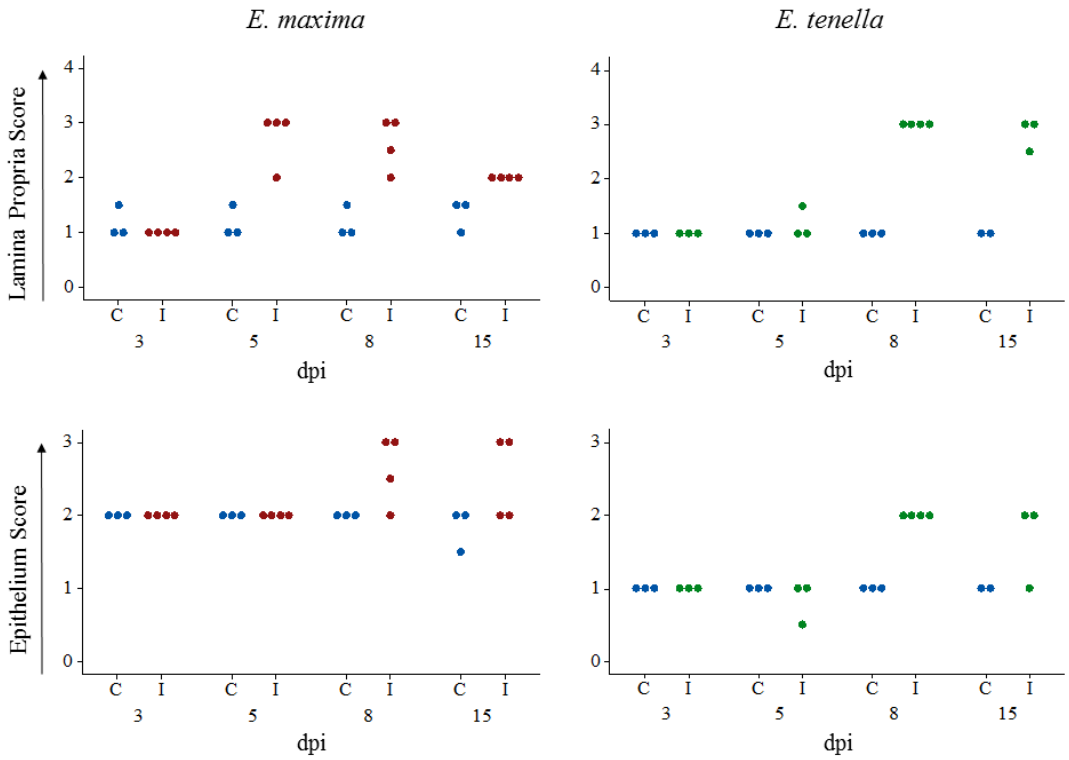

Figure 4-14: CD8α^{+} cells in the jejunum of *E. maxima*-infected and mid-caecum of *E. tenella*-infected Ross 308 broilers. Three-week-old Ross 308 broilers were orally inoculated with 2 x 10^{3} oocysts of either *E. maxima* or *E. tenella* and samples collected for histology at 3, 5, 8 and 15 dpi. Cell populations were scored as in section 2.6.2. Control (C) tissues are shown in blue and infected (I) tissues are shown in red for *E. maxima* and green for *E. tenella* infection. Each individual dot represents data from one bird.

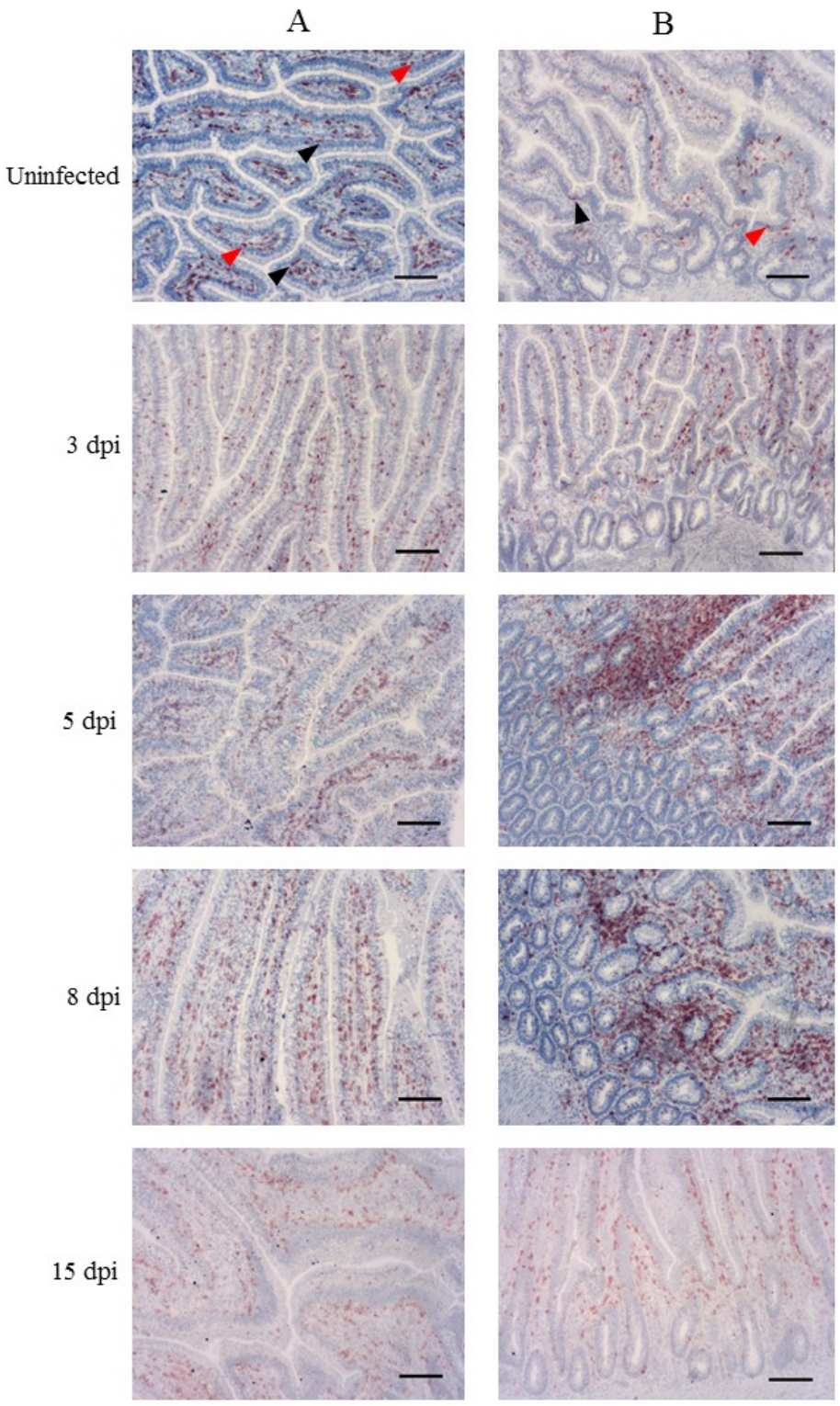

Figure 4-15: CD8α$^+$ cells in the villi (A) and crypts (B) in the jejunum of *E. maxima*-infected Ross 308 broilers. Three-week-old birds were orally inoculated with 2 x 10^3 *E. maxima* oocysts and samples collected for histology at 3, 5, 8 and 15 dpi. Red AEC stain indicates CD8α$^+$ cells. Black and red arrowheads indicate rounded and flattened CD8α$^+$ IELs respectively. Bars represent 100 µm.

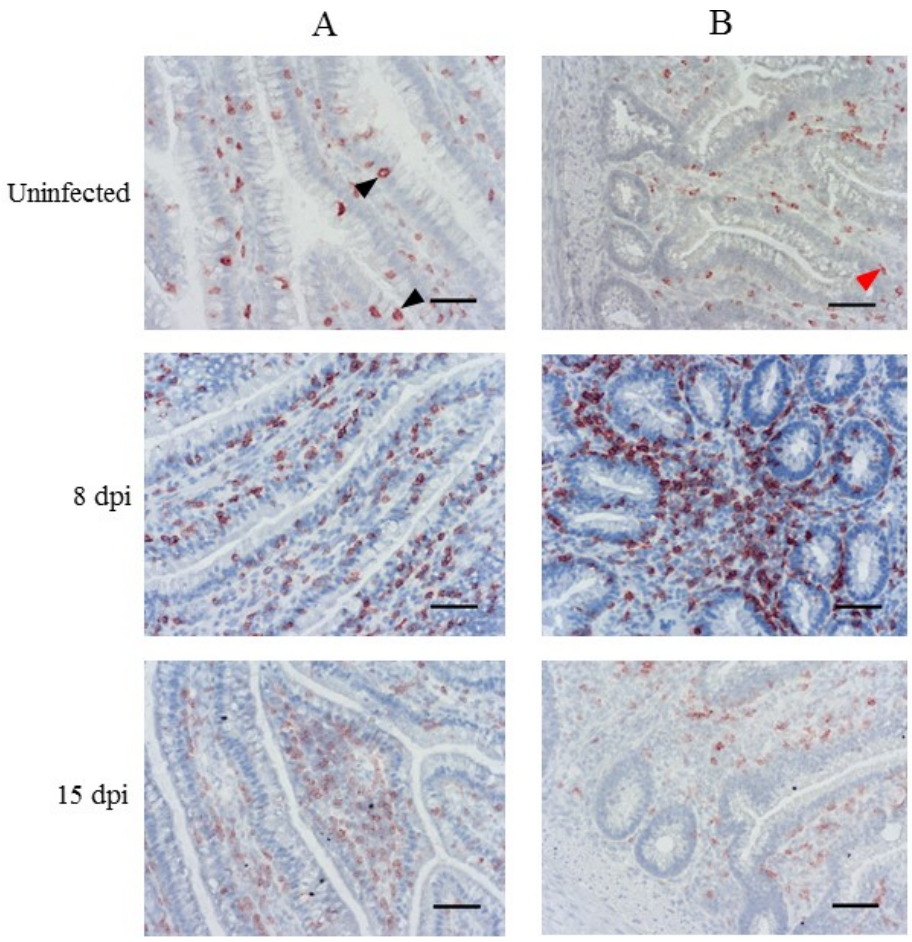

Figure 4-16: Increased CD8α$^+$ IELs in the villi (A) and crypts (B) in the jejunum of *E. maxima*-infected Ross 308 broilers. Three-week-old birds were orally inoculated with 2 x 10^3 *E. maxima* oocysts and samples collected for histology at 3, 5, 8 and 15 dpi. Red AEC stain indicates CD8α$^+$ cells. Black and red arrowheads indicate rounded and flattened CD8α$^+$ IELs respectively. Bars represent 50 µm.

In control caeca, CD8α$^+$ cells were both LPLs and IELs and similar morphological and spatial characteristics were observed to that as in the jejunum. The majority of CD8α$^+$ cells in control caeca were observed throughout the villi and in the areas of the crypts closer to the bases of the villi. Following *E. tenella* infection, both CD8α$^+$ LPLs and IELs were increased in the caeca compared to controls at 8 and 15 dpi (Figure 4-14, Figure 4-17 and Figure 4-18). At 8 dpi, in all birds CD8α$^+$ cell clusters were present in the lamina propria and at 15 dpi, in two of three birds CD8α$^+$ cell clusters were present (Figure 4-14 and Figure 4-17).

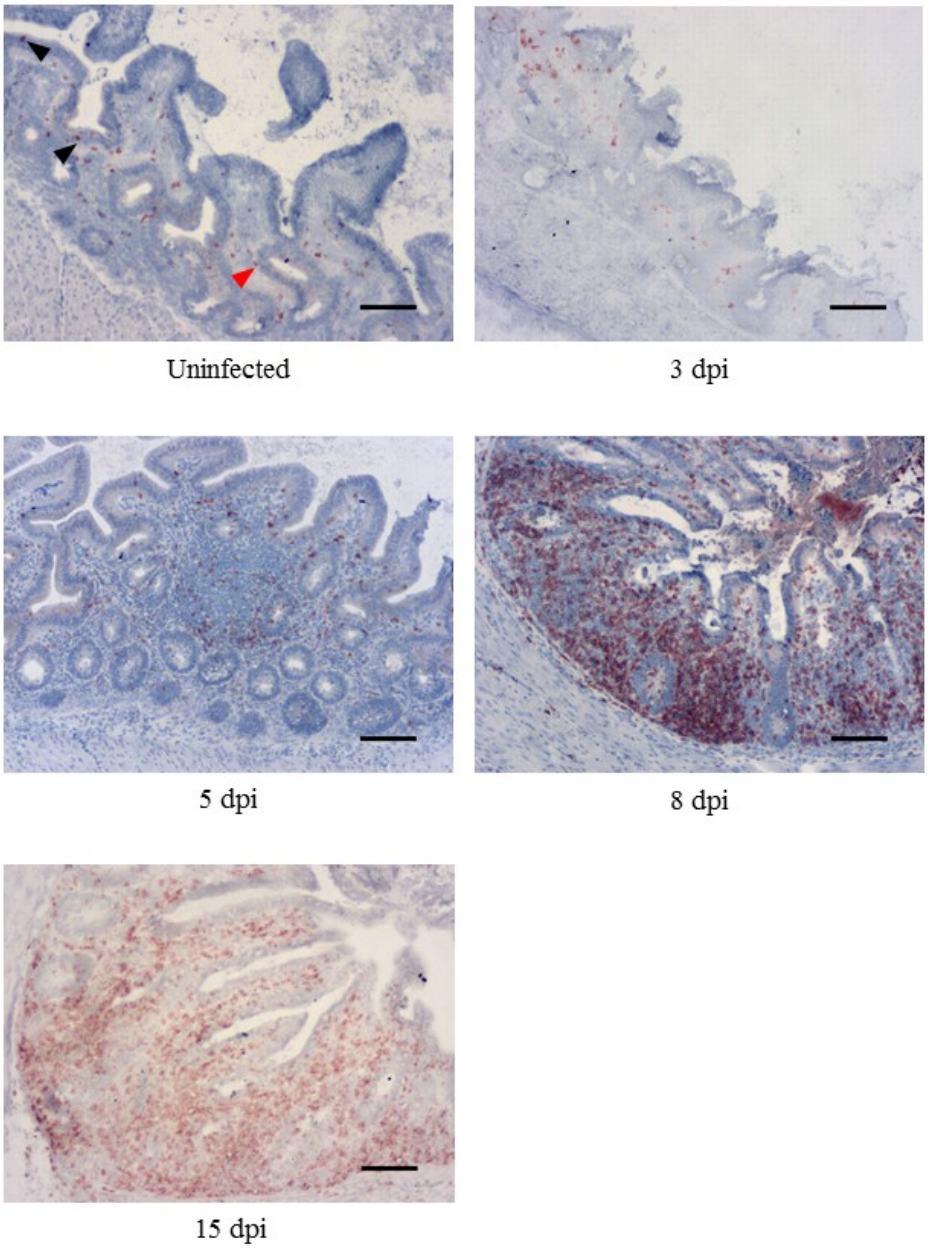

Figure 4-17: CD8α$^+$ cells in the mid-caecum of *E. tenella*-infected Ross 308 broilers. Three-week-old birds were orally inoculated with 2 x 10^3 *E. tenella* oocysts and samples collected for histology at 3, 5, 8 and 15 dpi. Red AEC stain indicates CD8α$^+$ cells. Black and red arrowheads indicate rounded and flattened CD8α$^+$ IELs respectively. Bars represent 100 μm.

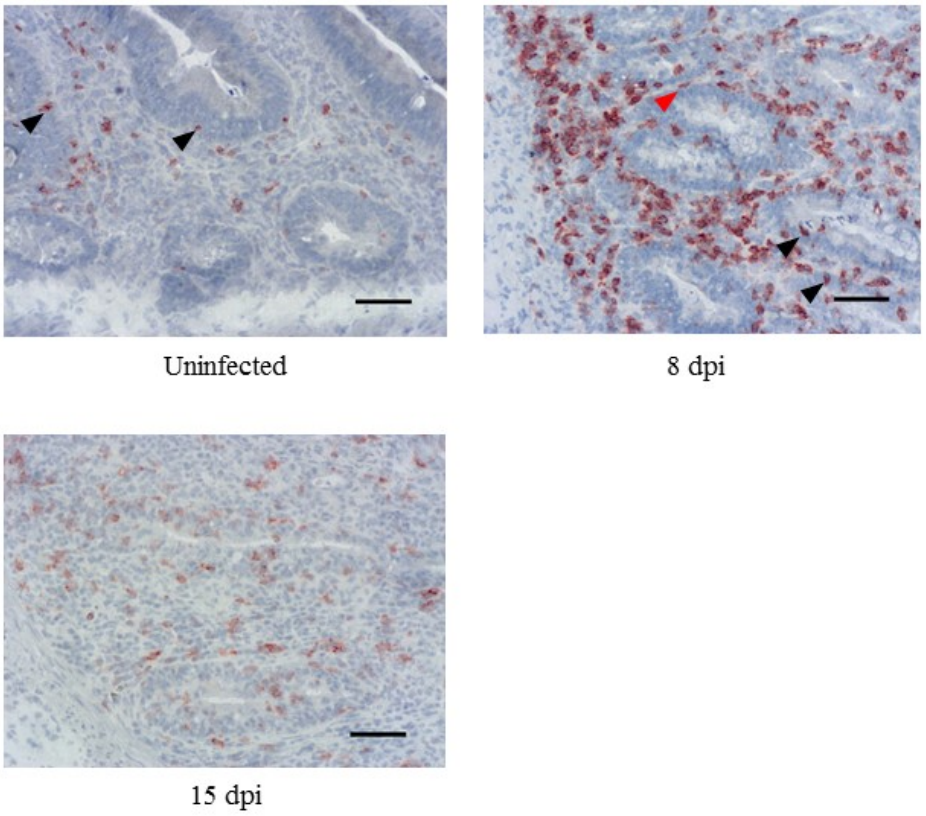

Figure 4-18: Increased CD8α$^+$ IELs in the mid-caecum of *E. tenella*-infected Ross 308 broilers. Three-week-old birds were orally inoculated with 2 x 10^3 *E. tenella* oocysts and samples collected for histology at 3, 5, 8 and 15 dpi. Red AEC stain indicates CD8α$^+$ cells. Black and red arrowheads indicate rounded and flattened CD8α$^+$ IELs respectively. Bars represent 50 µm.

In control jejunum, the majority of TCRγδ$^+$ cells were IELs and some were LPLs. The TCRγδ$^+$ LPLs were mostly situated below the epithelium in both the crypt and villi areas of the jejunum. TCRγδ$^+$ IELs were slightly larger in size than LPLs although both TCRγδ$^+$ LPLs and IELs were rounded in shape. Following *E. maxima* infection, increased numbers of TCRγδ$^+$ LPLs and IELs were observed when compared to control tissues in some, but not all, the birds at 5, 8 and 15 dpi (Figure 4-19, Figure 4-20 and Figure 4-21). No clusters of TCRγδ$^+$ LPLs were observed at any time point following *E. maxima* infection (Figure 4-19 and Figure 4-20).

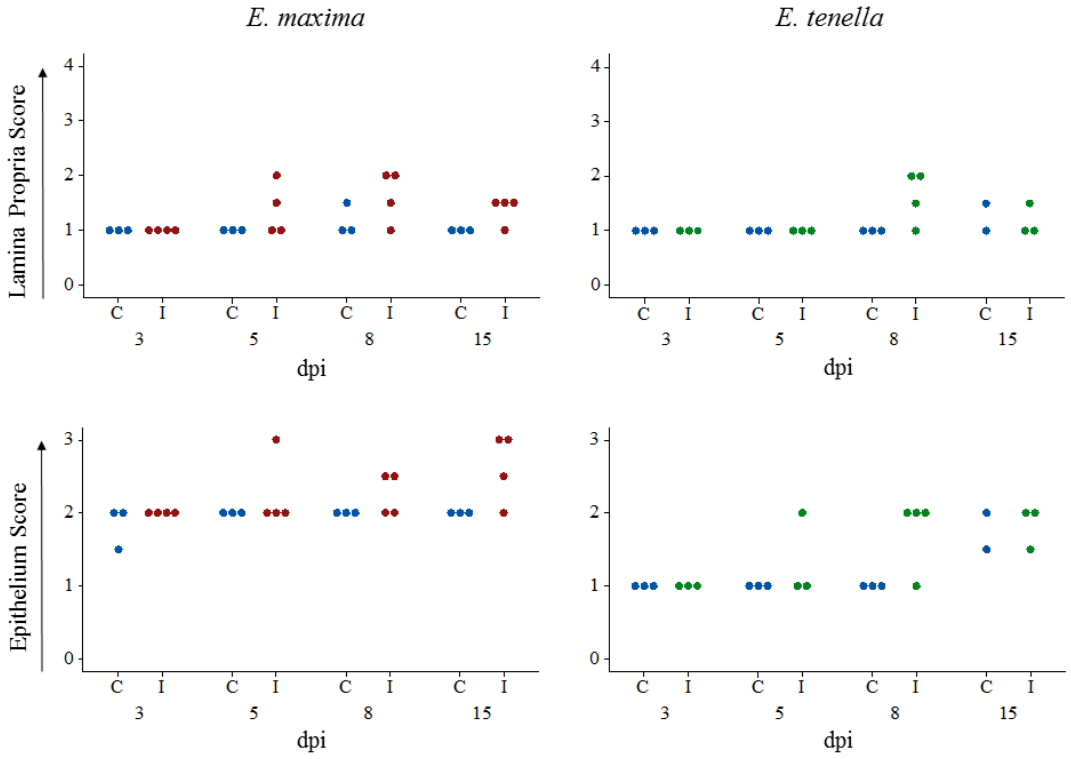

Figure 4-19: TCRγδ$^+$ cells in the jejunum of *E. maxima*-infected and mid-caecum of *E. tenella*-infected Ross 308 broilers. Three-week-old Ross 308 broilers were orally inoculated with 2 x 10^3 oocysts of either *E. maxima* or *E. tenella* and samples collected for histology at 3, 5, 8 and 15 dpi. Cell populations were scored as in section 2.6.2. Control (C) tissues are shown in blue and infected (I) tissues are shown in red for *E. maxima* and green for *E. tenella* infection. Each individual dot represents data from one bird.

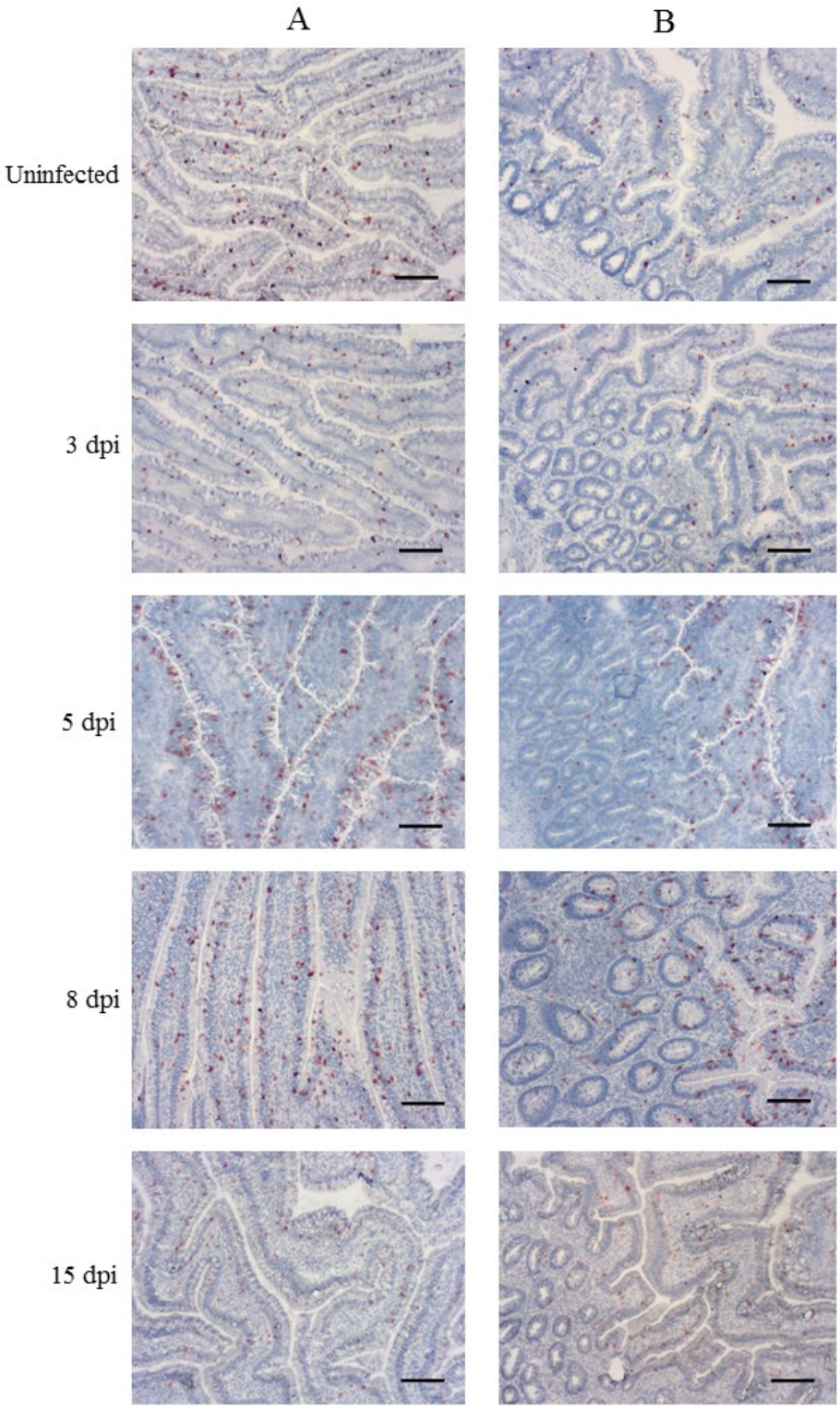

Figure 4-20: TCRγδ$^+$ cells in the villi (A) and crypts (B) in the jejunum of *E. maxima*-infected Ross 308 broilers. Three-week-old birds were orally inoculated with 2 x 10^3 *E. maxima* oocysts and samples collected for histology at 3, 5, 8 and 15 dpi. Red AEC stain indicates TCRγδ$^+$ cells. Bars represent 100 μm.

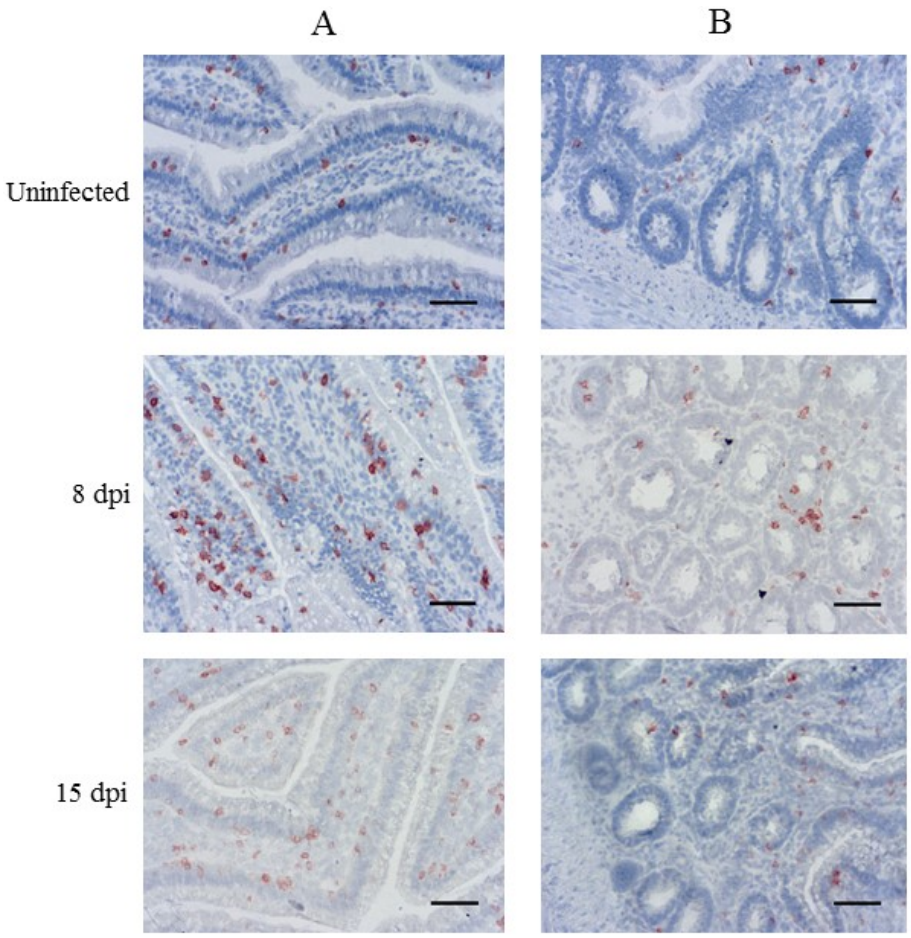

Figure 4-21: Increased TCRγδ$^+$ IELs in the villi (A) and crypts (B) in the jejunum of *E. maxima*-infected Ross 308 broilers. Three-week-old birds were orally inoculated with 2 x 10^3 *E. maxima* oocysts and samples collected for histology at 3, 5, 8 and 15 dpi. Red AEC stain indicates TCRγδ$^+$ cells. Bars represent 50 µm.

In control caeca, TCRγδ$^+$ LPLs were scattered throughout the lamina propria of both crypt and villi regions although, as in jejunum control tissues, many were found closer to the epithelium. TCRγδ$^+$ IELs were scattered throughout the epithelium. Both TCRγδ$^+$ LPLs and IELs in the caecum exhibited a rounded morphology. Following *E. tenella* infection, increases in TCRγδ$^+$ LPL numbers were observed at 8 and 15 dpi onwards. As in the *E. maxima*-infected groups, increases to the TCRγδ$^+$ cell population was not observed in all of the birds. In the caecum of one bird at 5 dpi, increased numbers of TCRγδ$^+$ IELs were present whereas at 8 dpi, almost all birds had increased IELs bearing the TCRγδ marker (Figure 4-19 and Figure 4-23). At 15 dpi, an increase in TCRγδ$^+$ IELs was observed. No clusters of TCRγδ$^+$ LPLs were observed at any time point following *E. tenella* infection (Figure 4-19 and Figure 4-22).

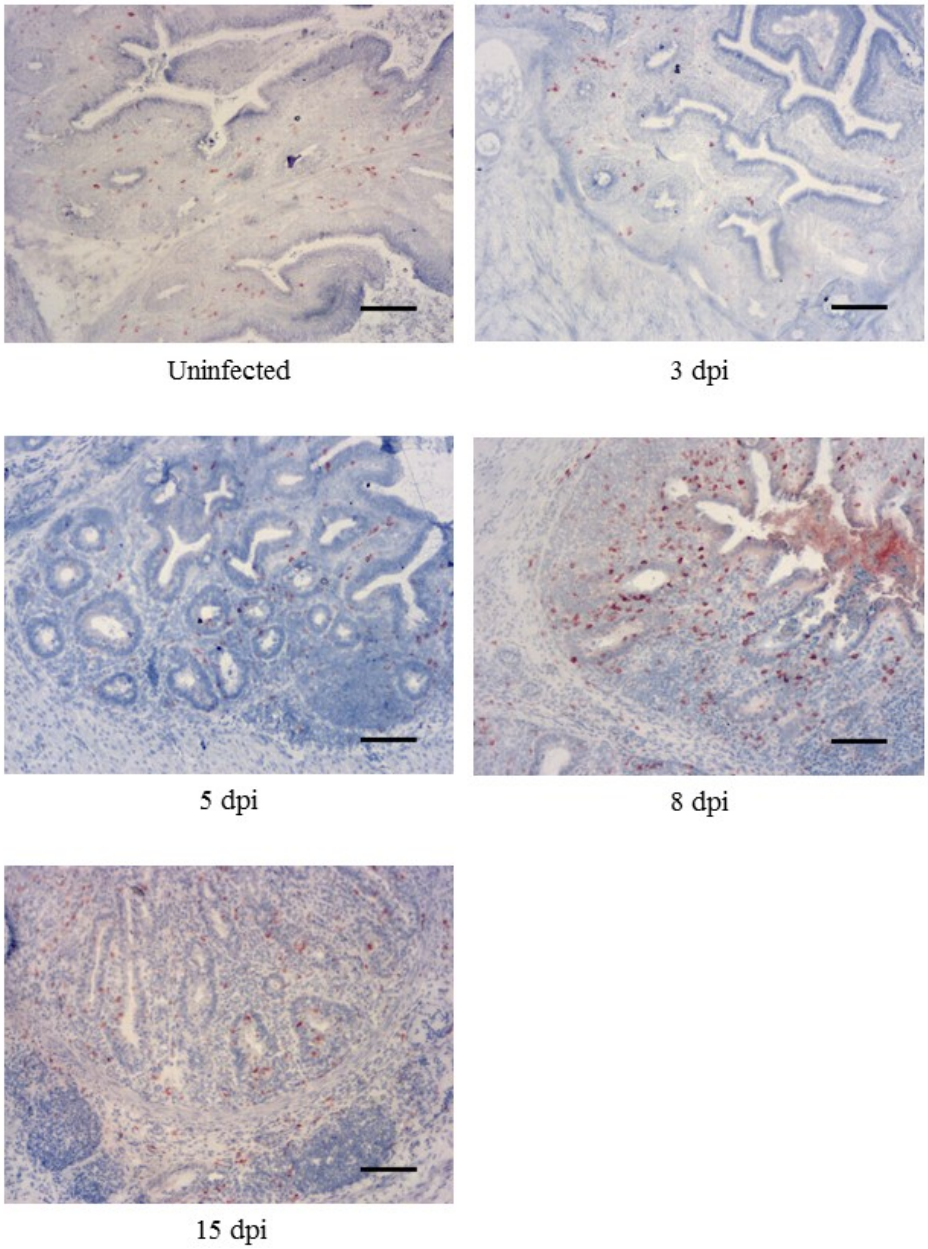

Figure 4-22: TCR$\gamma\delta^+$ cells in the mid-caecum of *E. tenella*-infected Ross 308 broilers. Three-week-old birds were orally inoculated with 2 x 10^3 *E. tenella* oocysts and samples collected for histology at 3, 5, 8 and 15 dpi. Red AEC stain indicates TCR$\gamma\delta^+$ cells. Bars represent 100 µm.

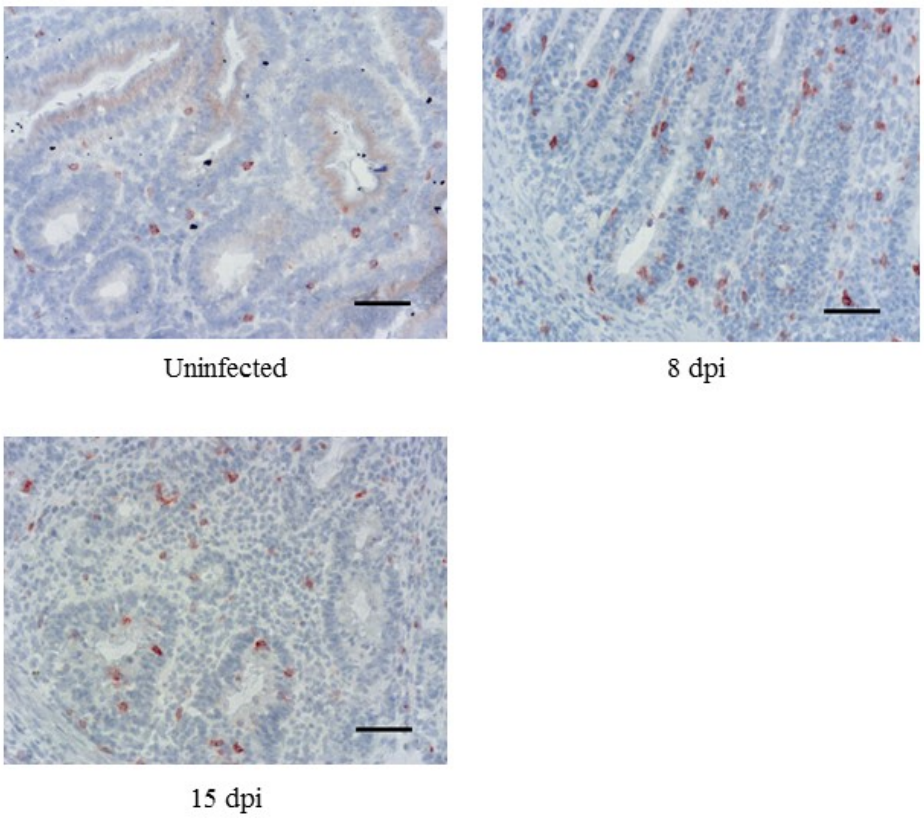

Figure 4-23: Increased TCRγδ$^+$ IELs in the mid-caecum of *E. tenella*-infected Ross 308 broilers. Three-week-old birds were orally inoculated with 2 x 10^3 *E. tenella* oocysts and samples collected for histology at 3, 5, 8 and 15 dpi. Red AEC stain indicates TCRγδ$^+$ cells. Bars represent 50 μm.

In jejunum from control birds, TCRαβ$_1^+$ cells were scattered throughout both the lamina propria and the epithelium, mainly in the villi and areas of the crypts close to the base of the villi. Many of the TCRαβ$_1^+$ LPLs were rounded and many were located close to the epithelium. The majority of TCRαβ$_1^+$ IELs were rounded although a few were observed to be flattened along the basal membrane. Following *E. maxima* infection, very small increases in TCRαβ$_1^+$ LPL numbers were seen in the lamina propria of three birds at 3 dpi. A more pronounced increase in TCRαβ$_1^+$ cells was observed from 5 dpi onwards. At 5 (in one of four birds) and 8 (in three of four birds) dpi, clusters of TCRαβ$_1^+$ LPLs could be seen in the jejunum. At 15 dpi, no clusters of TCRαβ$_1^+$ LPLs were present in the jejunum following *E. maxima* infection, however the numbers of TCRαβ$_1^+$ LPLs was still increased compared to controls. No changes were observed to the TCRαβ$_1^+$ IEL population of the jejunum following *E. maxima* infection (Figure 4-24 and Figure 4-25).

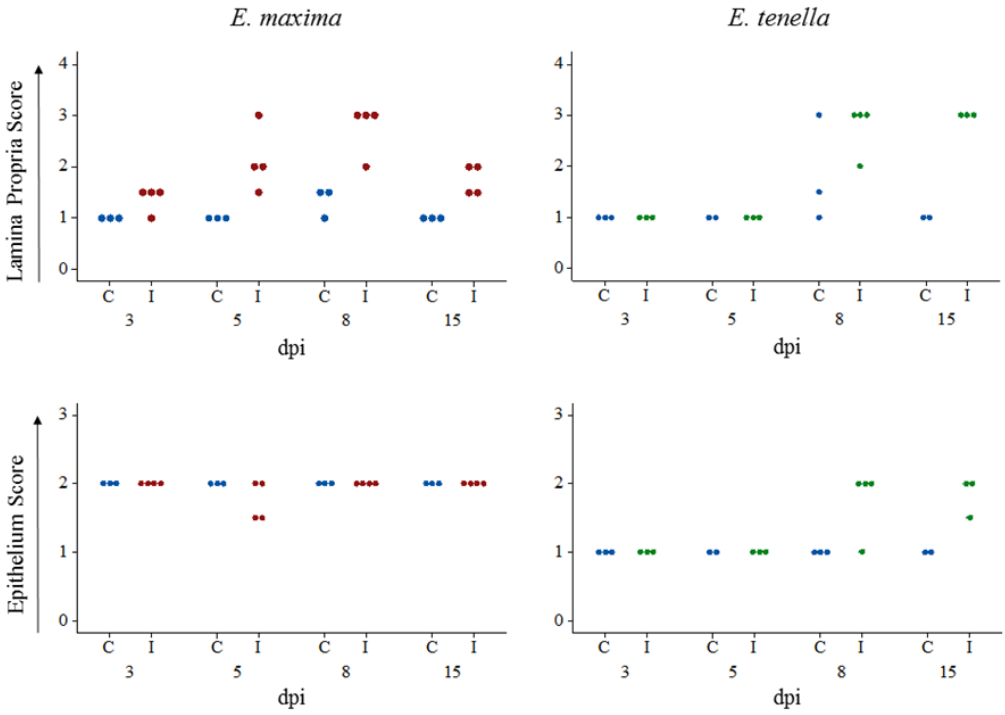

Figure 4-24: TCRαβ$_1^+$ cells in the jejunum of E. maxima-infected and mid-caecum of *E. tenella*-infected Ross 308 broilers. Three-week-old Ross 308 broilers were orally inoculated with 2 x 10^3 oocysts of either *E. maxima* or *E. tenella* and samples collected for histology at 3, 5, 8 and 15 dpi. Cell populations were scored as in section 2.6.2. Control (C) tissues are shown in blue and infected (I) tissues are shown in red for *E. maxima* and green for *E. tenella* infection. Each individual dot represents data from one bird.

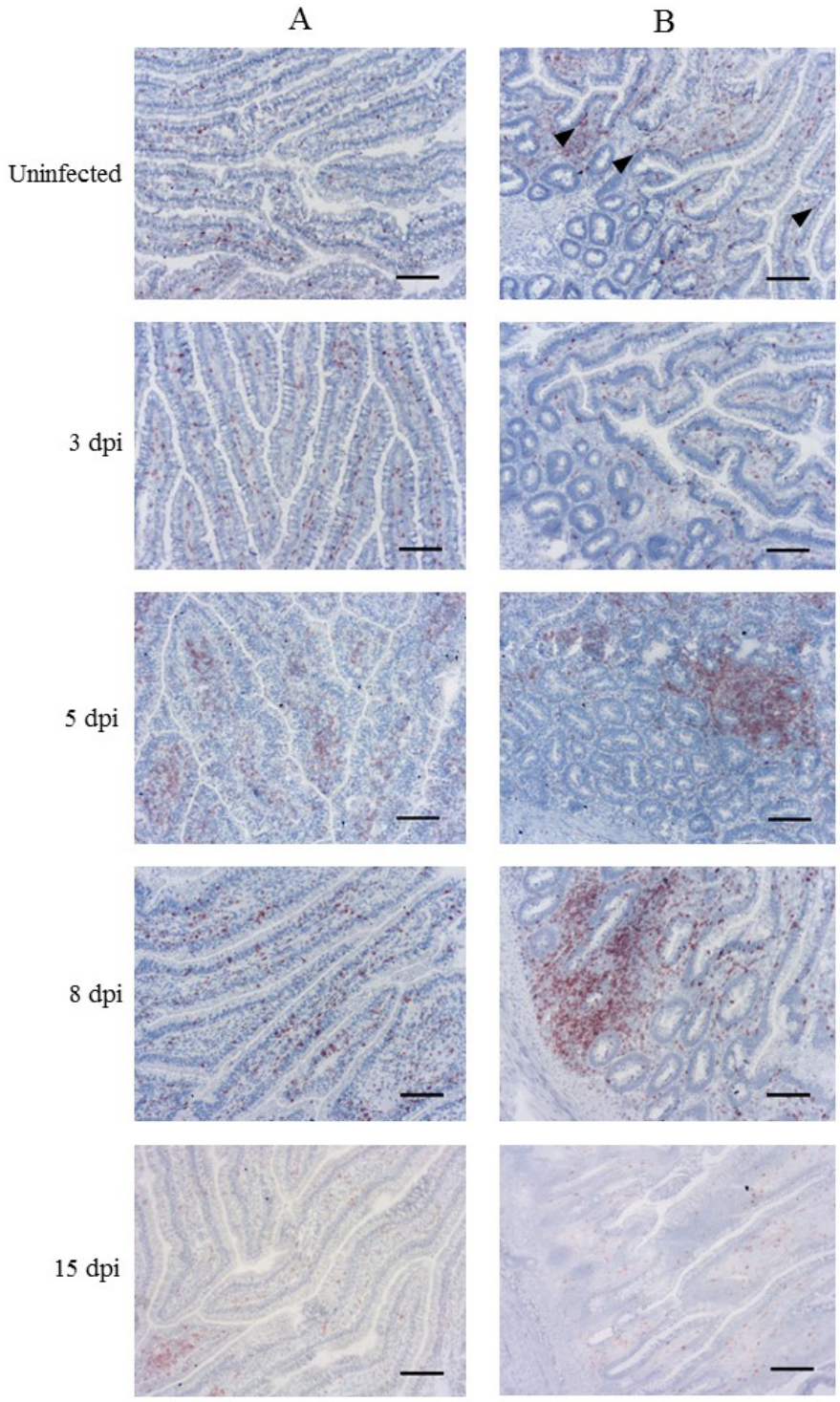

Figure 4-25: TCR$\alpha\beta_1^+$ cells in the villi (A) and crypts (B) in the jejunum of *E. maxima*-infected Ross 308 broilers. Three-week-old birds were infected with 2 x 10^3 *E. maxima* oocysts and samples collected for histology at 3, 5, 8 and 15 dpi. Red AEC stain indicates TCR$\alpha\beta_1^+$ cells. Black arrowheads indicate TCR$\alpha\beta_1^+$ IELs which were flattened along the basal membrane. Bars represent 100 μm.

In the caeca of uninfected birds, TCR$\alpha\beta_1^+$ cells were evenly scattered throughout the lamina propria and the epithelium of crypts and villi. Following *E. tenella* infection, the number of TCR$\alpha\beta_1^+$ LPLs increased at 8 and 15 dpi and clusters of TCR$\alpha\beta_1^+$ LPLs were observed in the crypts in almost all birds at 8 dpi and all birds at 15 dpi (Figure 4-24 and Figure 4-26). In contrast to infection with *E. maxima*, infection with *E. tenella* resulted in an increase to the number of TCR$\alpha\beta_1^+$ IELs in the caecum in almost all birds at 8 and all birds at 15 dpi (Figure 4-24 and Figure 4-27).

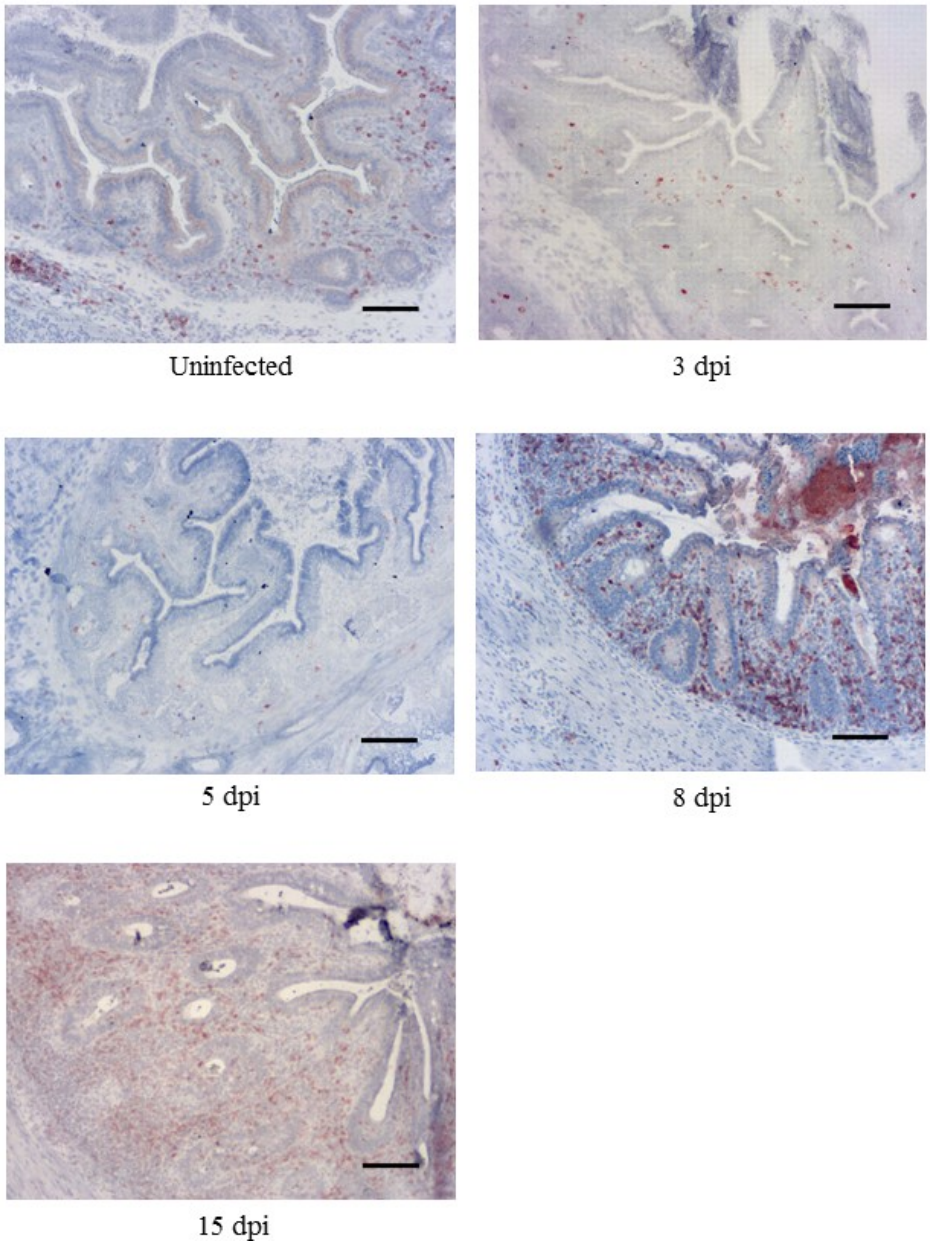

Figure 4-26: TCR$\alpha\beta_1^+$ cells in the mid-caecum of *E. tenella*-infected Ross 308 broilers. Three-week-old birds were orally inoculated with 2 x 10^3 *E. tenella* oocysts and samples collected for histology at 3, 5, 8 and 15 dpi. Red AEC stain indicates TCR$\alpha\beta_1^+$ cells. Bars represent 100 µm.

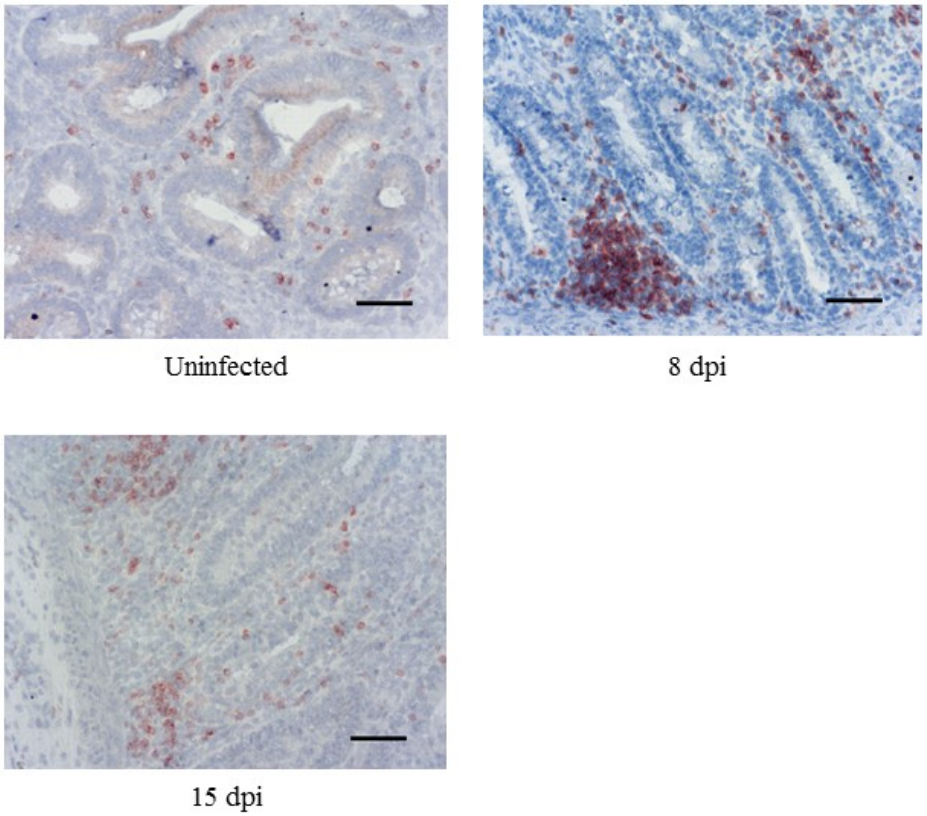

Figure 4-27: Increased TCR$\alpha\beta_1^+$ IELs in the mid-caecum of *E. tenella*-infected Ross 308 broilers. Three-week-old birds were orally inoculated with 2 x 10^3 *E. tenella* oocysts and samples collected for histology at 3, 5, 8 and 15 dpi. Red AEC stain indicates TCR$\alpha\beta_1^+$ cells. Bars represent 50 μm.

At no time point examined were TCR$\alpha\beta_2^+$ cells observed in the gut in either infected or control birds (Figure 4-28). The TCR3 antibody was successfully used to detect TCR$\alpha\beta_2^+$ cells in the spleen (data not shown).

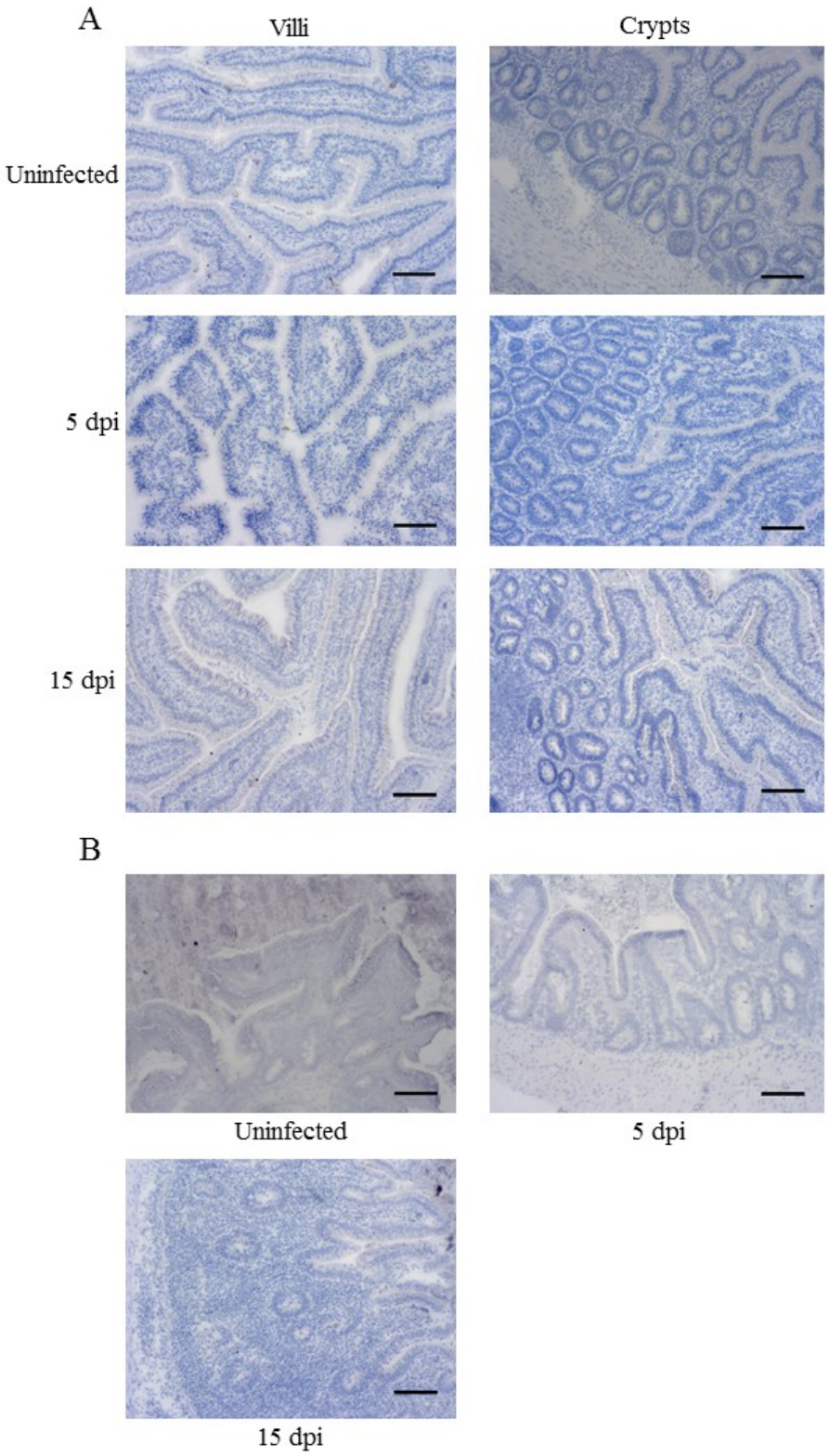

Figure 4-28: TCR$\alpha\beta_2^+$ cells in the villi and crypts of the jejunum (A) and in the caecum (B) of *E. maxima*- and *E. tenella*- infected Ross 308 broilers. Three-week-old birds were orally inoculated with 2 x 10^3 *E. maxima* or *E. tenella* oocysts and samples collected for histology at 5 and 15 dpi. Bars represent 100 µm.

In control jejunum, $CD25^+$ cells were located scattered throughout the lamina propria and the epithelium in both the crypt and villi regions. $CD25^+$ IELs were either rounded in shape or elongated and pressed against the basal membrane. Overall, following *E. maxima* infection, no change was observed to the $CD25^+$ cell population within the jejunum, either in the lamina propria or the epithelium (Figure 4-29 and Figure 4-30).

In control caeca, $CD25^+$ cells were mainly LPLs although some were IELs in both the crypts and villi. In contrast to *E. maxima* infection, *E. tenella* infection resulted in clusters of $CD25^+$ LPLs in the crypts in one of three birds at 3 dpi and two of three birds at 5 dpi. At 8 and 15 dpi, no $CD25^+$ cell clusters were observed but there was an increase to the number of $CD25^+$ LPLs present compared to controls. No changes were seen to the population of $CD25^+$ IELs following *E. tenella* infection (Figure 4-29 and Figure 4-31).

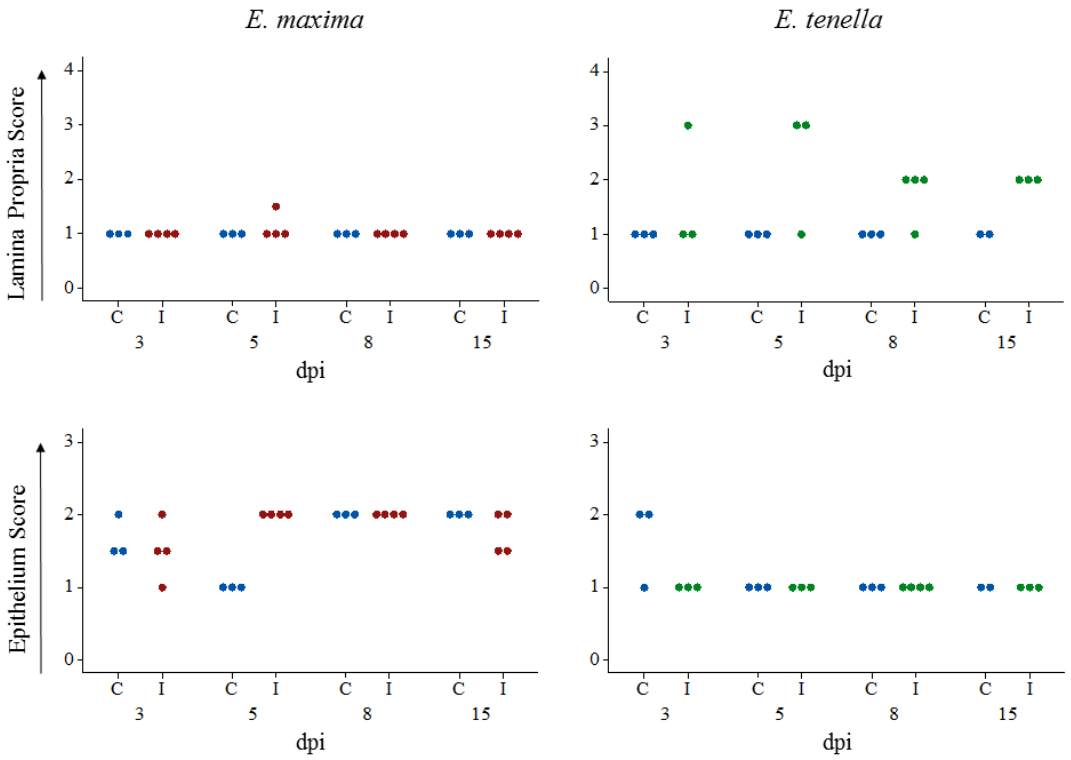

Figure 4-29: CD25^{+} cells in the jejunum of E. maxima-infected and mid-caecum of *E. tenella*-infected Ross 308 broilers. Three week old Ross 308 broilers were orally inoculated with 2 x 10^{3} oocysts of either *E. maxima* or *E. tenella* and samples collected for histology at 3, 5, 8 and 15 dpi. Cell populations were scored as in section 2.6.2. Control (C) tissues are shown in blue and infected (I) tissues are shown in red for *E. maxima* and green for *E. tenella* infection. Each individual dot represents data from one bird.

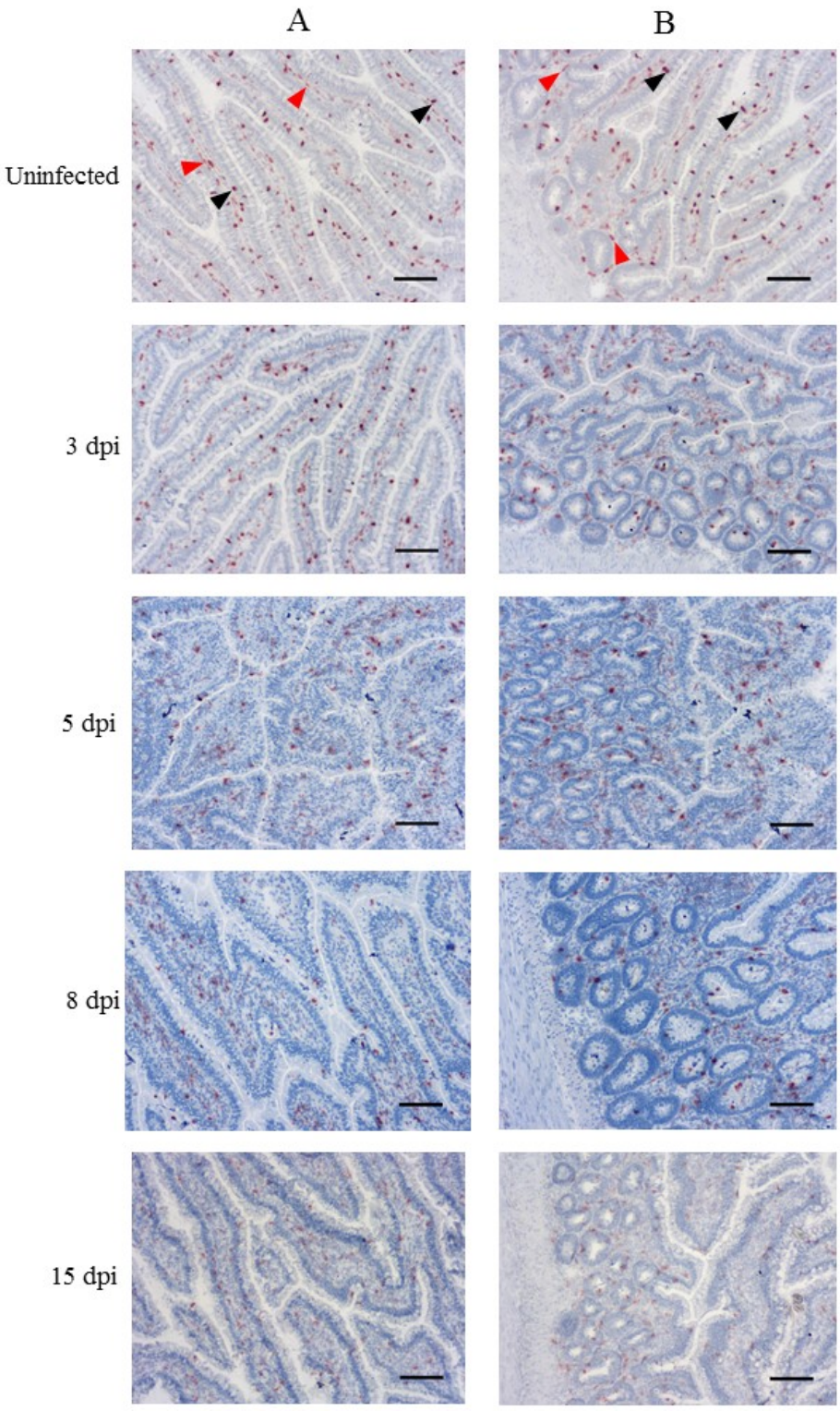

Figure 4-30: $CD25^+$ cells in the villi (A) and crypts (B) in the jejunum of *E. maxima*-infected Ross 308 broilers. Three-week-old birds were orally inoculated with 2 x 10^3 *E. maxima* oocysts and samples collected for histology at 3, 5, 8 and 15 dpi. Red AEC stain indicates $CD25^+$ cells. Black and red arrowheads indicate rounded and elongated $CD25^+$ IELs respectively. Bars represent 100 µm.

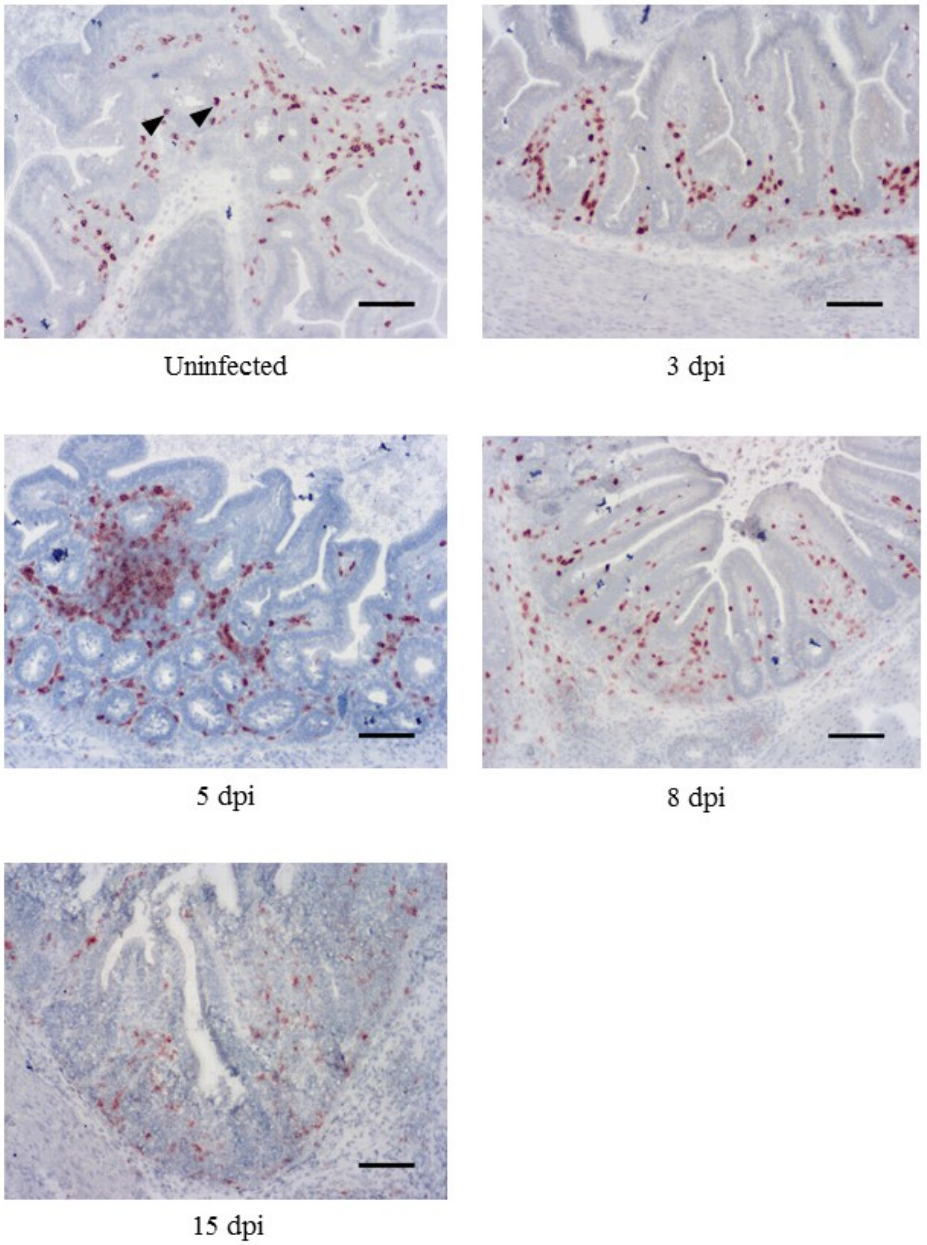

Figure 4-31: $CD25^+$ cells in the mid-caecum of *E. tenella*-infected Ross 308 broilers. Three-week-old birds were orally inoculated with 2 x 10^3 *E. tenella* oocysts and samples collected for histology at 3, 5, 8 and 15 dpi. Red AEC stain indicates $CD25^+$ cells. Black arrowheads indicate rounded $CD25^+$ IELs. Bars represent 100 µm.

In control jejunum, chB6$^+$ cells were mostly localised throughout the epithelium and very few cells were present in the lamina propria of the crypts and villi. Following *E. maxima* infection, increased chB6$^+$ LPLs in the jejunum were observed in one bird at 5 dpi and almost all birds at 8 dpi. In addition to this at 8 dpi, in one bird chB6$^+$ clusters were observed. By 15 dpi, chB6$^+$ clusters were identified in most birds and in one of the birds there was no change to the chB6$^+$cell population (Figure 4-32 and Figure 4-33). At 15 dpi, a germinal centre was observed as a cluster of tightly packed chB6$^+$ cells (Figure 4-33). Increased chB6$^+$ IELs were observed in the jejunum from 5 dpi onwards following *E. maxima* infection (Figure 4-32 and Figure 4-34).

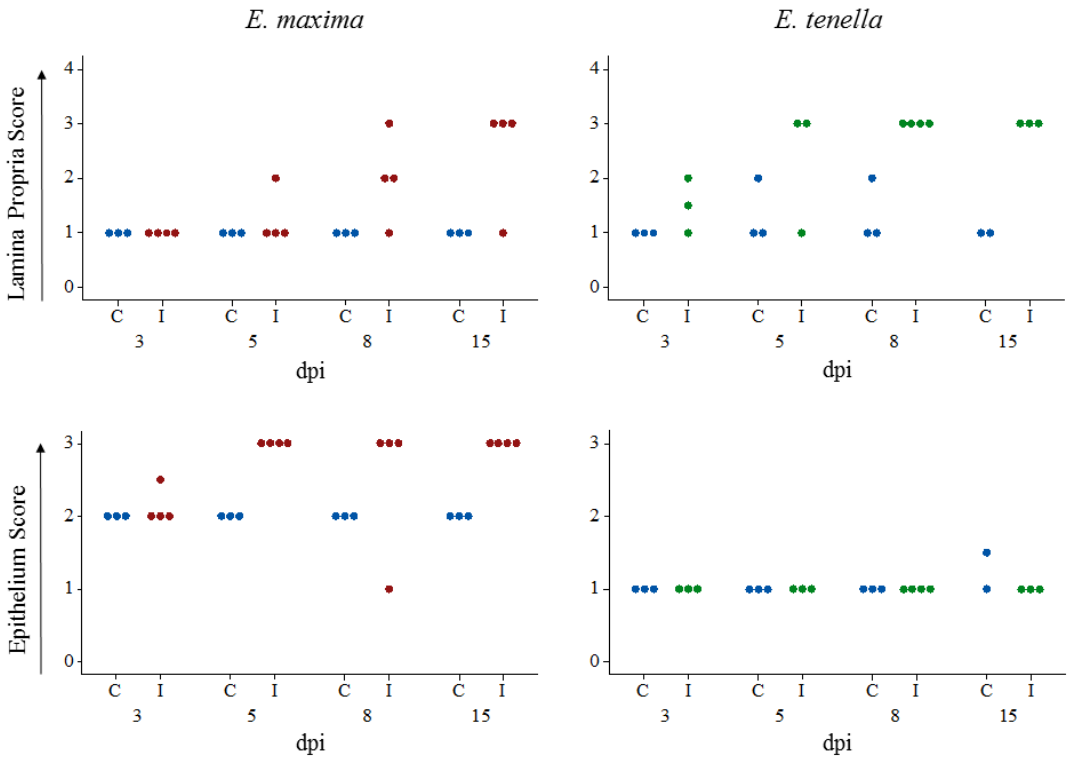

Figure 4-32: ChB6^{+} cells in the jejunum of *E. maxima*-infected and mid-caecum of *E. tenella*-infected Ross 308 broilers. Three week old Ross 308 broilers were orally inoculated with 2 x 10^{3} oocysts of either *E. maxima* or *E. tenella* and samples collected for histology at 3, 5, 8 and 15 dpi. Cell populations were scored as in section 2.6.2. Control (C) tissues are shown in blue and infected (I) tissues are shown in red for *E. maxima* and green for *E. tenella* infection. Each individual dot represents data from one bird.

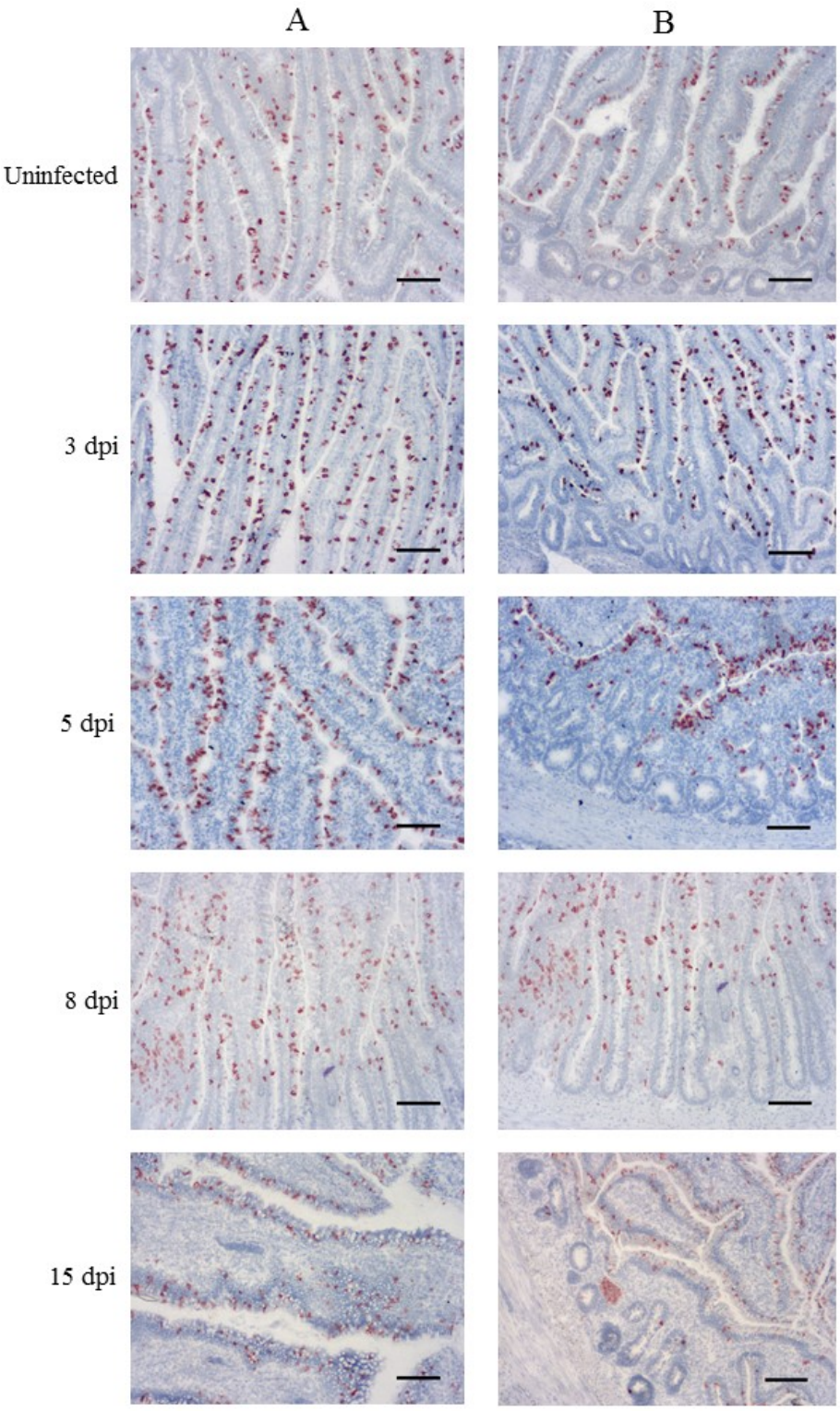

Figure 4-33: ChB6$^+$ cells in the villi (A) and crypts (B) in the jejunum of *E. maxima*-infected Ross 308 broilers. Three-week-old birds were orally inoculated with 2 x 10^3 *E. maxima* oocysts and samples collected for histology at 3, 5, 8 and 15 dpi. Red AEC stain indicates chB6$^+$ cells. Bars represent 100 μm.

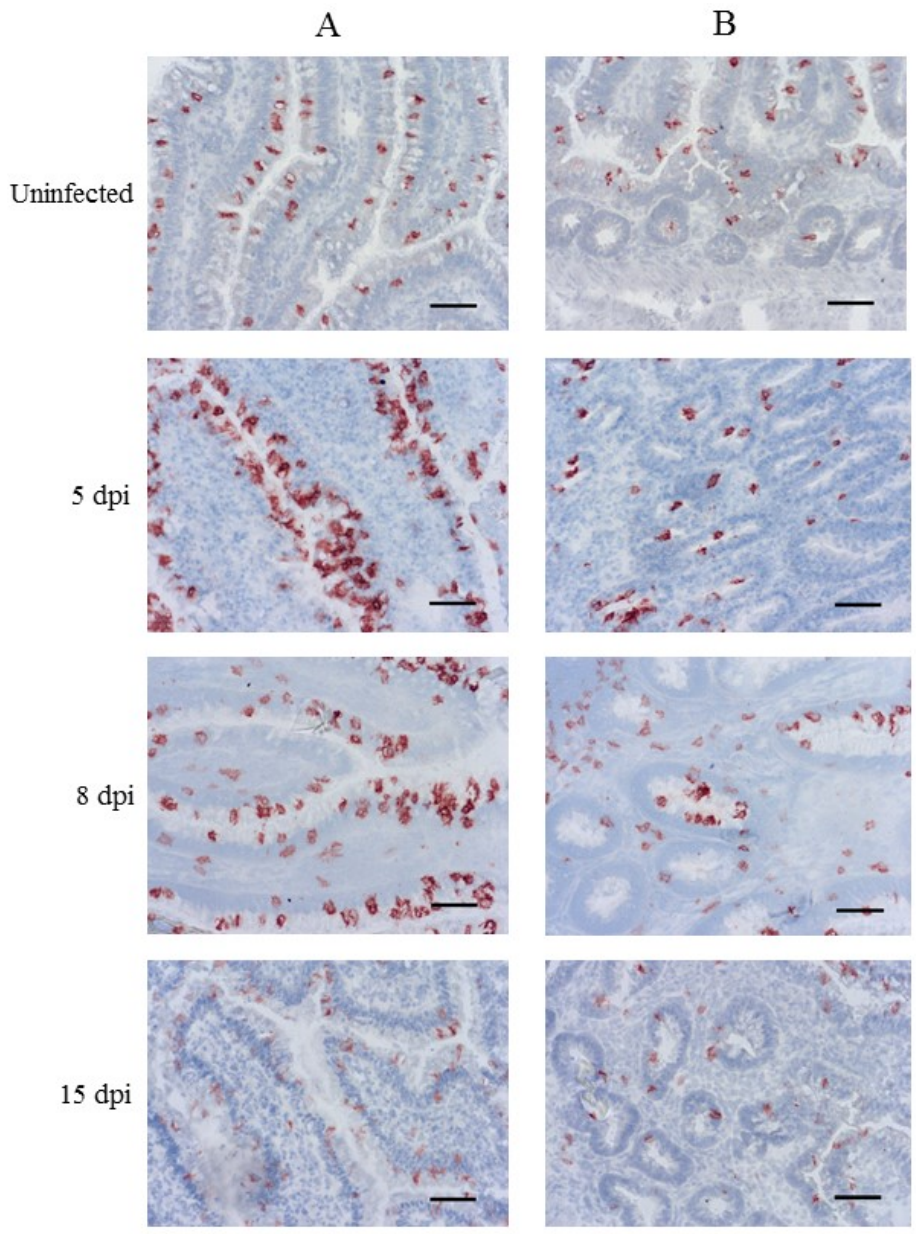

Figure 4-34: Increased chB6^{+} IELs in the villi (A) and crypts (B) in the jejunum of *E. maxima*-infected Ross 308 broilers. Three-week-old birds were orally inoculated with 2 x 10^{3} *E. maxima* oocysts and samples collected for histology at 3, 5, 8 and 15 dpi. Red AEC stain indicates chB6^{+} cells. Bars represent 50 µm.

In control caeca, chB6$^+$ cells were mostly IELs and very few were LPLs. Following *E. tenella* infection, an increase in chB6$^+$ LPLs was observed at 3 dpi in almost all birds. Clusters of chB6$^+$ LPLs were observed in almost all birds at 5 dpi and in all birds at 8 and 15 dpi. No changes were observed to chB6$^+$ IEL numbers in *E. tenella*-infected caeca compared to uninfected birds (Figure 4-32 and Figure 4-35). Germinal centres containing chB6$^+$ cells were also observed in one of the tissues at 15 dpi (as shown in Figure 4-35).

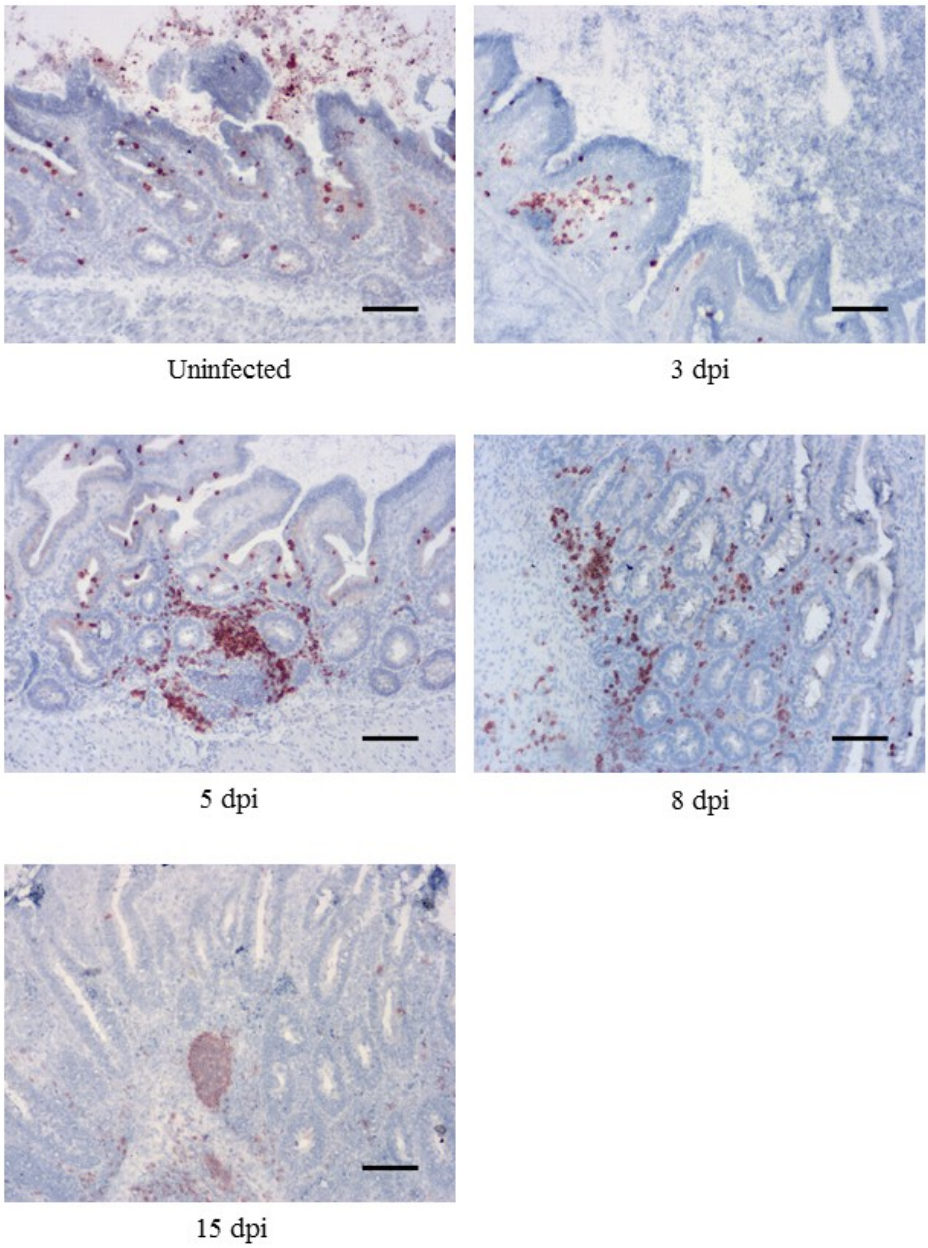

Figure 4-35: ChB6$^+$ cells in the mid-caecum of *E. tenella*-infected Ross 308 broilers. Three-week-old birds were orally inoculated with 2 x 10^3 *E. tenella* oocysts and samples collected for histology at 3, 5, 8 and 15 dpi. Red AEC stain indicates chB6$^+$ cells. Bars represent 100 µm.

In control jejunum and caecum, MRC1L-B$^+$ cells were present in the lamina propria of the crypts and villi in both jejunum and caecum tissues. No MRC1L-B$^+$ cells were observed in the epithelium. MRC1L-B$^+$ cells were of mixed morphology, some were more rounded in shape and others were more elongated. Overall, no major changes to the total number of MRC1L-B$^+$ cells occurred following either *E. maxima* or *E. tenella* infection with the exception of 8 dpi where there was a slight increase in MRC1L-B$^+$ cell numbers during infection with both *Eimeria* species (Figure 4-36, Figure 4-37 and Figure 4-38).

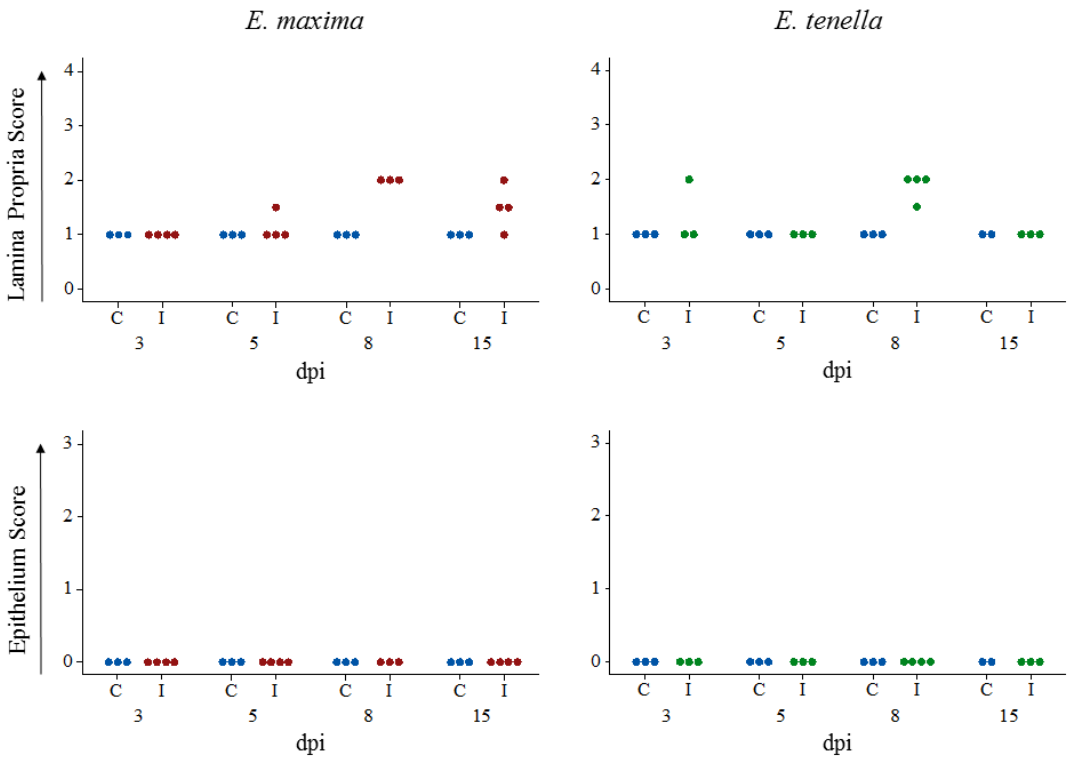

Figure 4-36: MRC1L-B^{+} (KUL01^{+}) cells in the jejunum of *E. maxima*-infected and mid-caecum of *E. tenella*-infected Ross 308 broilers. Three week old Ross 308 broilers were orally inoculated with 2 x 10^{3} oocysts of either *E. maxima* or *E. tenella* and samples collected for histology at 3, 5, 8 and 15 dpi. Cell populations were scored as in section 2.6.2. Control (C) tissues are shown in blue and infected (I) tissues are shown in red for *E. maxima* and green for *E. tenella* infection. Each individual dot represents data from one bird.

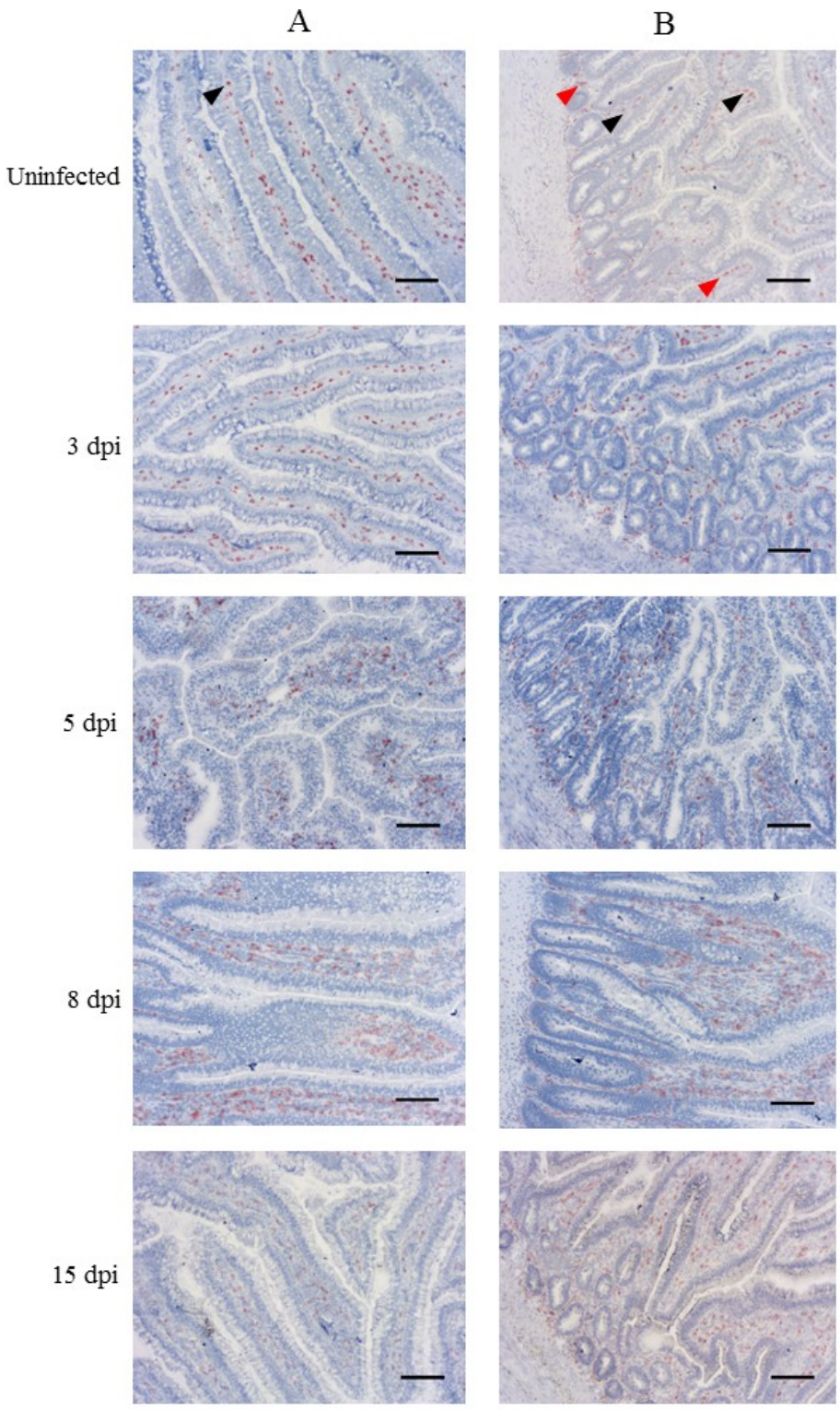

Figure 4-37: MRC1L-B$^+$ (KUL01$^+$) cells in the villi (A) and crypts (B) in the jejunum of *E. maxima*-infected Ross 308 broilers. Three-week-old birds were orally inoculated with 2 x 10^3 *E. maxima* oocysts and samples collected for histology at 3, 5, 8 and 15 dpi. Red AEC stain indicates MRC1L-B$^+$ cells. Black and red arrowheads indicate rounded and elongated MRC1L-B$^+$ LPLs respectively. Bars represent 100 µm.

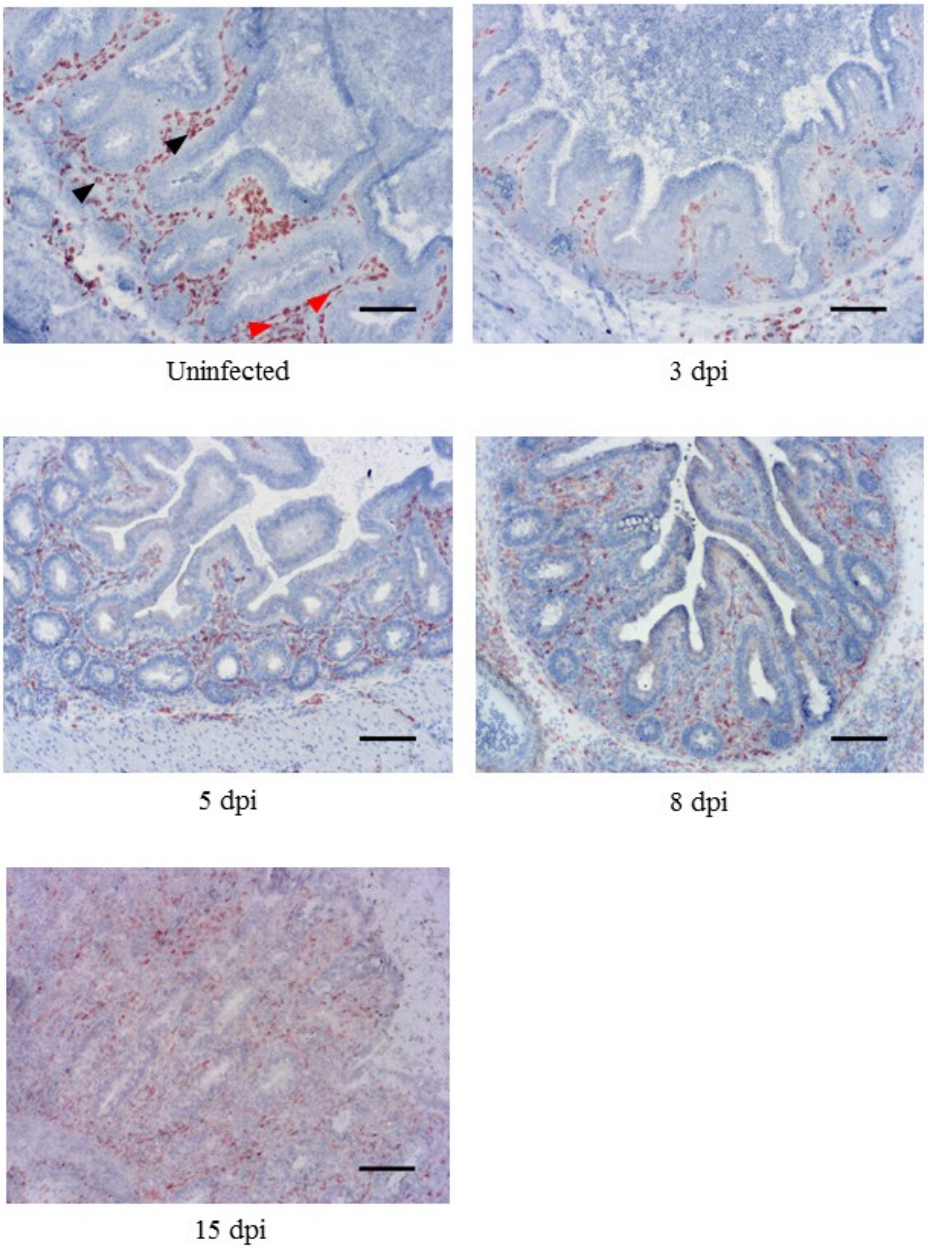

Figure 4-38: MRC1L-B$^+$ (KUL01$^+$) cells in the mid-caecum of *E. tenella*-infected Ross 308 broilers. Three-week-old birds were orally inoculated with 2 x 10^3 *E. tenella* oocysts and samples collected for histology at 3, 5, 8 and 15 dpi. Red AEC stain indicates MRC1L-B$^+$ cells. Black and red arrowheads indicate rounded and elongated MRC1L-B$^+$ LPLs respectively. Bars represent 100 µm.

Tim4^{+} cells were present within the lamina propria but not epithelium in both the jejunum and caeca of control birds. Staining against Tim4 produced results similar to that of MRC1L-B^{+} cells; Tim4^{+} cells were evenly scattered throughout the lamina propria and many were located underneath the epithelium in the villi and crypt regions. Tim4^{+} cells were a mixture of rounded and elongated morphologies. Overall, no changes were observed to the number of Tim4^{+} cells in the lamina propria of the jejunum after *E. maxima* infection or caeca after *E. tenella* infection.

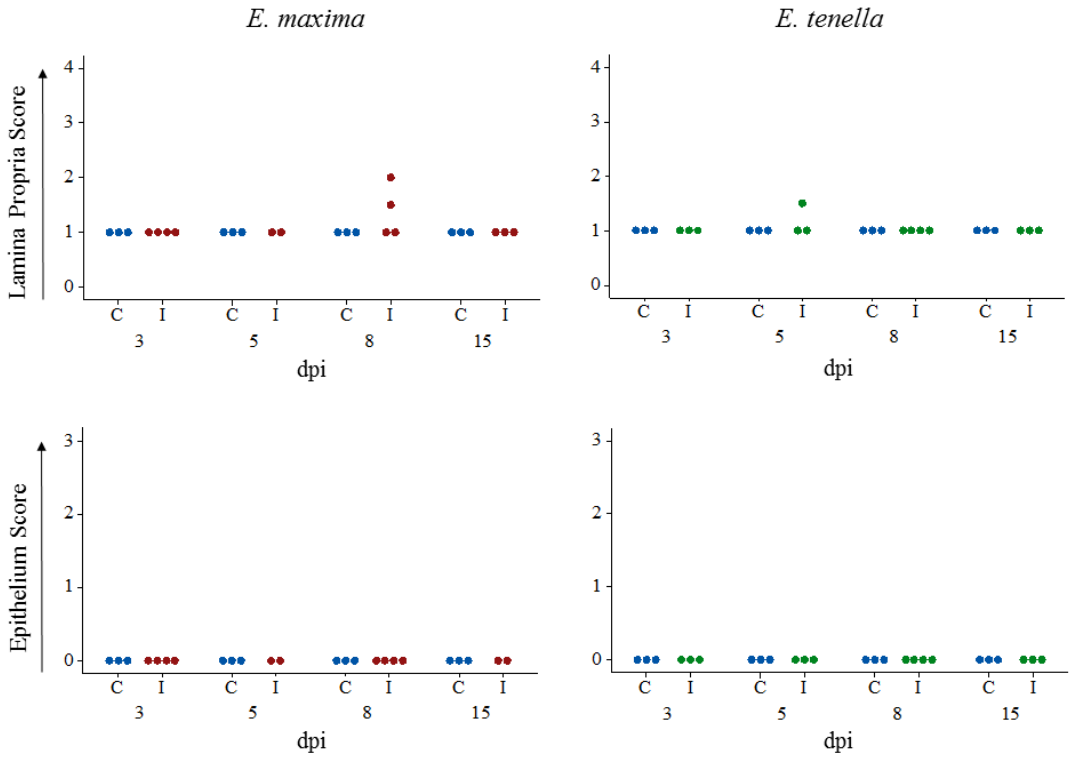

Figure 4-39: Tim4^{+} cells in the jejunum of *E. maxima*-infected and mid-caecum of *E. tenella*-infected Ross 308 broilers. Three week old Ross 308 broilers were orally inoculated with 2 x 10^{3} oocysts of either *E. maxima* or *E. tenella* and samples collected for histology at 3, 5, 8 and 15 dpi. Cell populations were scored as in section 2.6.2. Control (C) tissues are shown in blue and infected (I) tissues are shown in red for *E. maxima* and green for *E. tenella* infection. Each individual dot represents data from one bird.

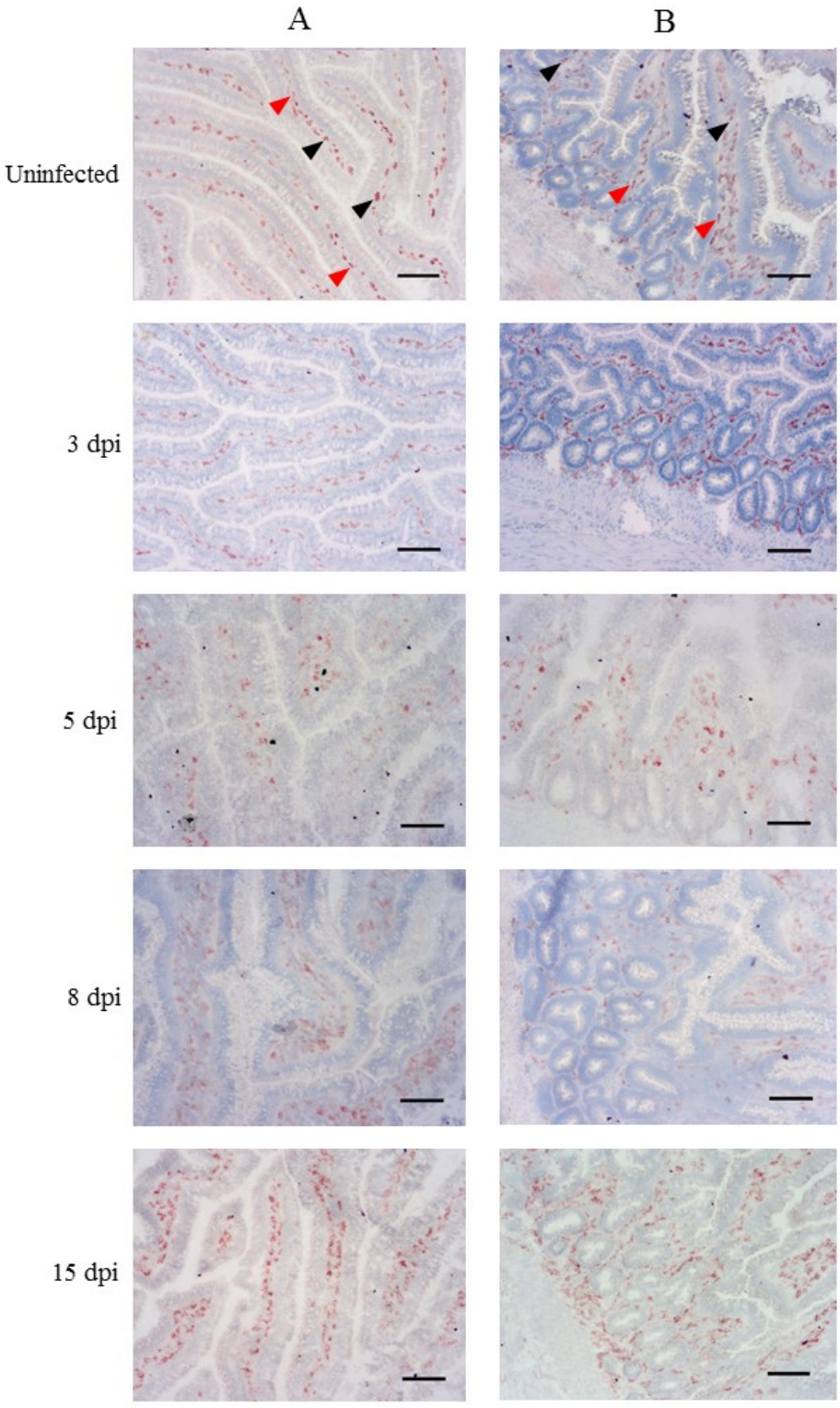

Figure 4-40: Tim4^{+} cells in the villi (A) and crypts (B) in the jejunum of *E. maxima*-infected Ross 308 broilers. Three-week-old birds were orally inoculated with 2 x 10^{3} *E. maxima* oocysts and samples collected for histology at 3, 5, 8 and 15 dpi. Red AEC stain indicates Tim4^{+} cells. Black and red arrowheads indicate rounded and elongated Tim4^{+} LPLs respectively. Bars represent 100 µm.

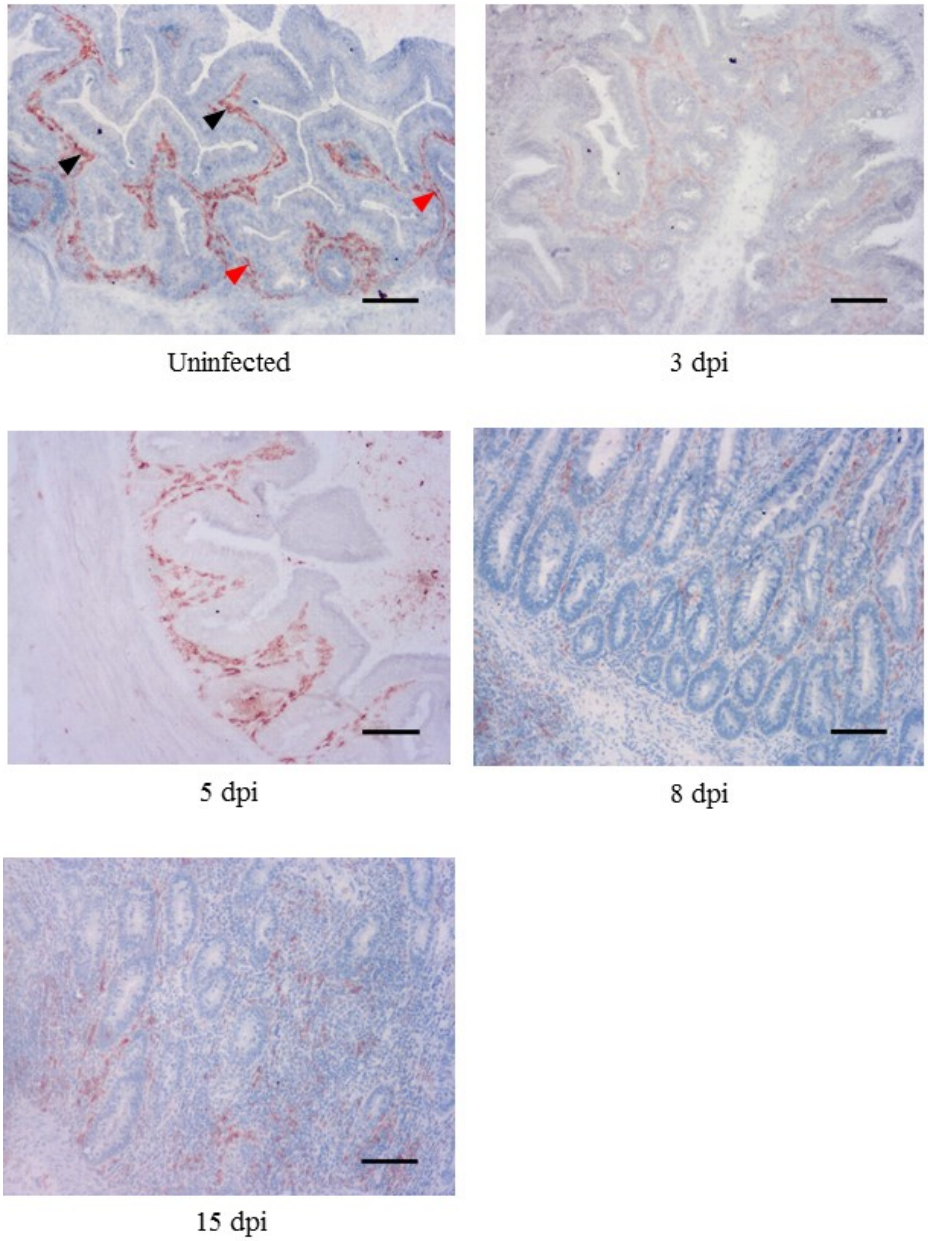

Figure 4-41: Tim4^{+} cells in the mid-caecum of *E. tenella*-infected Ross 308 broilers. Three-week-old birds were orally inoculated with 2 x 10^{3} *E. tenella* oocysts and samples collected for histology at 3, 5, 8 and 15 dpi. Red AEC stain indicates Tim4^{+} cells. Black and red arrowheads indicate rounded and elongated Tim4^{+} LPLs respectively. Bars represent 100 µm.

To investigate the relationship between changes in cell subpopulations and increased parasite genome copy numbers in the jejunum following *E. maxima* and caecum following *E. tenella* infection, the Spearman's rank correlation coefficient was calculated between parasite genome copy number and the scores assigned to descriptively quantify the cell populations in tissue sections of infected birds (Table 4-2).

A weak positive correlation was found between LPLs expressing either CD4, TCRγδ, CD25, MRC1L-B or Tim4 and a higher *E. maxima* genome copy number in the jejunum of infected birds although none of these correlations were statistically significant. However, increased abundance of $CD8\alpha^+$ and $TCR\alpha\beta_1^+$ LPLs was strongly correlated with increased *E. maxima* genome copy number and both were significant at $p<0.05$. ChB6 was negatively correlated with *E. maxima* genome copy number although not significantly. The $CD4^+$, $CD8\alpha^+$, $TCR\gamma\delta^+$ and $TCR\alpha\beta_1^+$ IEL populations were all negatively correlated with *E. maxima* genome copy number in the jejunum however none of these correlations were statistically significant. $CD25^+$ and $chB6^+$ IELs were both positively correlated with *E. maxima* genome copy number, although only $CD25^+$ IELs were strongly correlated and significant at $p<0.05$.

No variation was observed in the IEL populations expressing MRC1L-B or Tim4 in the jejunum during *E. maxima* infection and so the Spearman's rank correlation coefficients could not be calculated.

Table 4-2: Spearman's rank correlation coefficients between increased cell numbers and *Eimeria* parasite copy number in infected Ross 308 broilers. Correlation coefficients were calculated between scores assigned as detailed in section 2.6.2 and the parasite genome copy number per mg of host tissue. Shown is the Spearman's correlation coefficient for data of all infected birds (For all *E. maxima*-infected jejunum tissue sections n = 14. For *E. tenella*-infected caecum tissue sections n = 16 for CD4, CD8α, TCRγδ, TCRαβ$_1$, CD25 and chB6 stained sections, n = 15 for MRC1L-B stained sections and n = 13 for Tim4 stained sections) pooled from all time points. A coefficient value between -1 and 0 indicates a negative correlation, a coefficient value of 0 indicates no correlation and a coefficient value between 0 and 1 indicates a positive correlation. P values were calculated using the two-tailed t test and were considered significant at $p<0.05$ and highly significant at $p<0.001$.

Cell marker	*E. maxima*		*E. tenella*	
	LPLs	IELs	LPLs	IELs
CD4	0.432582 (p=0.094)	-0.445247 (p=0.083)	0.342259 (p=0.252)	-
CD8α	0.723458 (p<0.05)	-0.083031 (p=0.760)	-0.094616 (p=0.759)	-0.444951 (p=0.128)
TCRγδ	0.324555 (p=0.220)	-0.039112 (p=0.886)	0.370625 (p=0.213)	0.727533 (p<0.05)
TCRαβ$_1$	0.549201 (p<0.05)	-0.481688 (p=0.059)	0.427960 (p=0.145)	0.450927 (p=0.122)
CD25	0.329055 (p=0.213)	0.617903 (p<0.05)	0.239210 (p=0.431)	-
chB6	-0.149881 (p=0.580)	0.402823 (p=0.122)	0.712824 (p<0.05)	-
MRC1L-B	0.072503 (p=0.790)	-	0.073366 (p=0.812)	-
Tim4	0.427352 (p=0.099)	-	0.231455 (p=0.447)	-

During *E. tenella* infection, the population of LPLs expressing CD4, TCRγδ, TCRαβ$_1$, CD25, MRC1L-B or Tim4 were positively correlated with *E. tenella* genome copy number in the caecum although these correlations were not significant. However, the population of chB6$^+$ LPLs strongly correlated with *E. tenella* genome copy number and was significant ($p<0.05$). Of the LPL population during *E. tenella* infection, only CD8α$^+$ cells were negatively correlated with *E. tenella* genome copy number but this was not significant.

Both TCRγδ$^+$ and TCRαβ$_1^+$ IELs were more abundant in tissues containing higher *E. tenella* parasite genome copy numbers but of these only the correlation between TCRγδ$^+$ IELs and parasite genome copy number was significant. The CD8α$^+$ IEL population was negatively correlated with *E. tenella* genome copy number although this correlation was weak and not significant.

No changes to IELs expressing CD4, CD25, chB6, MRC1L-B or Tim4 was observed in the caecum during *E. tenella* infection and so Spearman's rank correlation coefficients could not be calculated.

4.3 Discussion

During the course of the Ross 308 commercial bird trial, the birds were housed on a litter pen. Consequently, once oocysts were shed following infection, birds were open to oocyst recycling. The *Eimeria* life cycle, from initial ingestion of *Eimeria* to excretion of unsporulated oocysts, lasts approximately 7-8 days (Al-Badri *et al.*, 2012) therefore birds were open to oocyst recycling from around 7 dpi. Following *E. maxima* infection, no further parasite genomic DNA was observed in the jejunum after 11 dpi (Figure 4-2), indicating that the broilers had developed immunity to re-infection with *E. maxima*. This supports previous studies where secondary challenge with homologous *E. maxima* strains induced protective immunity in chickens and fewer or no oocysts were excreted following secondary challenge with the same strain (Rose *et al.*, 1962; Smith *et al.*, 2002)

E. tenella parasite genomic DNA peaked at 6 dpi and was almost cleared by 11 dpi. The increase of *E. tenella* genomic DNA at 15 dpi is potentially a result of oocyst recycling and insufficient protective immunity to prevent re-infection caused by oocyst recycling. Upon *E. tenella* primary infection in White Leghorn chicken lines, immunity is built but does not give total protection and fewer oocysts are produced following secondary challenge than after the initial primary infection (Bumstead *et al.*, 1995).

Faecal oocyst counts have been used as an indicator of susceptibility or resistance to *Eimeria*, as a measure of the development of protective immunity and a method to quantify parasite replication (Bumstead *et al.*, 1995; Smith *et al.*, 2002; Rothwell *et al.*, 2004). In this experiment, the quantity of parasite genomic DNA present in the gut as measured by qPCR of a RAPD-SCAR marker was used as an indicator of

parasite replication in the gut following *Eimeria* infection. This method is found to be comparable with faecal oocyst count as an indicator of parasite replication, as both methods are strongly correlated with inoculation dose (Nolan *et al.*, 2015).

The main aim of this trial was to identify a Th17-mediated response in Ross 308 broilers during *E. maxima* or *E. tenella* infection. To detect changes to the expression of cytokines considered to be markers of a Th17 response (TGF-β4, IL-17A, IL-17F, IL-21, IL-22 and IL-23R) qRT-PCR was performed. The qRT-PCR data suggests that Th17 cells are unlikely to be involved in the immune response to *E. maxima* or *E. tenella* in Ross 308 broilers. IL-21 was the only Th17-associated cytokine to become up-regulated following infection and the lack of change to other Th17 mediators suggests that IL-21 is acting in another capacity than as a Th17 cytokine. In mammals, functions of IL-21 include enhancing $CD4^{+}TCR\alpha\beta^{+}$ T cell proliferation and differentiation into Th17 cells (Wei *et al.*, 2007), inhibiting DC maturation (Brandt *et al.*, 2003) and enhancing expansion of and cytotoxicity in $CD8^{+}$ (Zeng *et al.*, 2005) and NK cells (Smyth *et al.*, 2005; Coquet *et al.*, 2007), memory B cell formation (Nagumo *et al.*, 2009), B cell differentiation into plasma cells (Ozaki *et al.*, 2004; Ettinger *et al.*, 2005), regulating Ig production in B cells (Ozaki *et al.*, 2002), enhancing phagocytic activities of macrophages (Ruckert *et al.*, 2007), inducing IL-10 production in $CD4^{+}$ and $CD8^{+}$ T cells (Spolski *et al.*, 2009) and suppression of IFN-γ in developing Th1 cells (Suto *et al.*, 2006). This raises the question of what function IL-21 may have during *Eimeria* infection. In the chicken, IL-21 is produced by $CD4^{+}$ and $TCR\alpha\beta_{1}^{+}$ cells (both stimulated and unstimulated) and stimulated $chB6^{+}$ and $CD8^{+}$ cells (Rothwell *et al.*, 2012). In mammals, IL-21 is also produced by Tfh, $CD8^{+}$ and NKT cells (Coquet *et al.*, 2007; Vogelzang *et al.*,

2008; Mittal *et al.*, 2012). $CD4^+$, $CD8\alpha^+$, $TCR\alpha\beta_1^+$ and $chB6^+$ cells increased in the jejunum following *E. maxima* and in the caecum following *E. tenella* infection and are the potential sources of increased *IL21* mRNA during infection. From this experiment, it appears that IL-21 may have roles in both innate and adaptive immunity to *Eimeria.* It is possible that the early IL-21 production seen here is at least partially responsible for the increased $CD4^+$ and $TCR\alpha\beta_1^+$ cell numbers observed at 5 and 8 dpi with *E. maxima* and *E. tenella*, or to the increased numbers of $CD8\alpha^+$ cells at 5 dpi onwards during *E. maxima* infection and at 8 dpi onwards following *E. tenella* infection. IL-21 may also be involved in the production of IL-10 during the innate phase of the immune response following *E. maxima* infection and in both the innate and adaptive phases following *E. tenella* infection. IL-21 produced during the innate response may also be influencing the phagocytic capacity of macrophages, although no changes were observed in the numbers of $MRC1L\text{-}B^+$ and $Tim4^+$ LPLs. IL-21 is not currently associated with the response to *Eimeria* infection in the chicken, however in a study by Gowen *et al.* (2008), recombinant *E. acervulina* profilin-like antigen induced immunoprophylaxis of Punta Toro virus in hamsters and *IL21* mRNA was increased in the spleen following exposure to the antigen. Additionally, IL-21 is important during the immune response to *T. gondii* infection. Lower IgG production, lower B cell numbers in the germinal centre, decreased $CD4^+$ and $CD8^+$ cell numbers and overall decreased IFN-γ production by T cells was observed in $IL\text{-}21^{-/-}$ mice during chronic *T. gondii* infection (Stumhofer *et al.*, 2013a). The same study also showed a reduction in IL-10-producing $CD4^+$ brain mononuclear cells in $IL\text{-}21^{-/-}$ mice although an increase in brain immunopathology was not observed whereas in a previous study of toxoplasmic

encephalitis in IL-10$^{-/-}$ mice, lethal brain immunopathology was observed (Wilson *et al.*, 2005) indicating that IL-21 is not important for IL-10 production during *T. gondii* infection. Furthermore, during *Plasmodium chabaudi* infection in mice it was shown that IL-21 produced by T cells was important to *P. chabaudi*-specific IgG production and development of memory B cells. Mice deficient in either IL-21 or IL-21R failed to clear infection with *P. chabaudi* and were not immune to re-infection (Pérez-Mazliah *et al.*, 2015).

To further characterise the immune response to *Eimeria* and identify other T helper subsets involved, *IFNG*, *IL13*, *IL10*, *IL2* and *IL15* were also measured by RT-qPCR. Following both *E. maxima* and *E. tenella* infection, *IFNG*, *IL10*, *IL2* and *IL21* mRNA was up-regulated in the jejunum and caecum following infection with respective *Eimeria* species. The level of expression of *IFNG*, *IL21* and *IL10* mRNA correlated positively with the quantity of parasite genome copy number in the gut during both *E. maxima* and *E. tenella* infection indicating that expression of these cytokines increases with parasite burden.

IFN-γ is proinflammatory and the hallmark cytokine of a Th1 response and has previously shown to be an important factor in immunity to *Eimeria* (Rose *et al.*, 1989) and this is further supported in this study. Early *IFNG* mRNA expression observed in this experiment (from 4 dpi during *E. maxima* and 5 dpi during *E. tenella* infection) is unlikely indicative of a Th1 response and more likely a component of the inflammatory response to the *Eimeria* parasite. The early *IFNG* observed is likely produced by innate cells such as NK cells and CD8α^{+} cytotoxic T cells. However, *IFNG* mRNA observed at later time points is likely the result of a

polarised Th1 response and the main source of IFN-γ is likely to be Th1 cells. IL-13 is considered a Th2-associated cytokine but its production is not limited to Th2 cells. In mammals, IL-13 has also been shown to be produced by NK, mast, Th1 and Th17 cells. *IL13* mRNA was unchanged following infection with either *Eimeria* species in this trial suggesting that the Th2 response does not have a role in the response to *Eimeria* infection.

IL2 mRNA was increased following *E. maxima* and *E. tenella* infection, although after infection with *E. maxima*, there was a delay in the up-regulation of *IL2* in comparison with *E. tenella* infection. A previous study showed upregulation of *IL2* mRNA in IELs following *E. acervulina* infection, but down-regulated following *E. tenella* infection (Hong *et al.*, 2006a). A further study found *IL2* mRNA was down-regulated in duodenum IELs following primary infection with *E. acervulina* but up-regulated following secondary infection (Choi *et al*., 2000). Similarly to mammalian IL-2, chicken IL-2 stimulates proliferation and activity of NK (Jansen *et al.*, 2010) and T cells. Chickens injected with IL-2 DNA had higher numbers of splenic TCRγδ$^+$ and CD8$^+$ cells (Choi *et al*., 2000). In this trial, IL-2 is unlikely to be the cause of increased CD8α$^+$ and TCRγδ$^+$ cells in the jejunum during *E. maxima* infection as IL-2 is not significantly up-regulated until after clusters of these cells appear, however, IL-2 may be a factor in shaping CD8α$^+$ and TCRγδ$^+$ responses during *E. tenella* infection. The *IL2* mRNA produced at 11 and 15 dpi with *E. maxima* may be contributing towards shaping the adaptive Th1 response.

Following both *E. maxima* and *E. tenella* infection, clusters of CD4$^+$, CD8α$^+$ and TCRαβ$_1^+$ cells were present in the lamina propria of birds in the jejunum and caecum

respectively. Following intra-caecal inoculation of *E. tenella* sporozoites, Vervelde *et al.* (1996) found that $CD4^+$ cells in the caecum were also mainly $TCR\alpha\beta_1^+$. Here, clusters of both $CD4^+$ and $TCR\alpha\beta_1^+$ IELs were observed following both *E. maxima* and *E. tenella* infection and a proportion of these are likely to be double positive for CD4 and $TCR\alpha\beta_1$. This study also found that immune chickens had higher numbers of $CD8\alpha^+TCR\alpha\beta_1^+$ cells compared to naïve chickens, which had higher numbers of $CD8\alpha^+TCR\alpha\beta_1^-$ cells.

No clusters of $TCR\gamma\delta^+$ cells were observed, however the numbers of these cells did increase in both the jejunum and caecum lamina propria following infection with *E. maxima* and *E. tenella*. $TCR\gamma\delta^+$ cells also increased in the epithelium of the jejunum and caecum following infection with *E. maxima* and *E. tenella.* The role of $TCR\gamma\delta^+$ cells in immunity to *Eimeria* is controversial. Rose *et al*. (1996) found that antibody-mediated depletion of $TCR\gamma\delta^+$ cells in mice infected with *E. vermiformis* did not have a significant impact on parasite replication however Smith *et al.* (2000b) found that $TCR\beta^{-/-}$ mice were more resistant to *E. vermiformis* infection than $TCR(\beta x\delta)^{-/-}$ mice. $TCR\gamma\delta^+$ cells have been implicated in resistance to *Eimeria* infection. *E. maxima*-resistant line C.B12 chickens had greater increases in $TCR\gamma\delta^+$ PBLs following *E. maxima* infection than susceptible line 15I (Bumstead *et al.*, 1995). Following *E. tenella* infection, *E. tenella*-resistant line SC chickens had greater increases in $CD4^+TCR\gamma\delta^+$ cells in the CT compared to that of *E. tenella*-susceptible line TK chickens, which had greater increases in the number of $CD8^+TCR\alpha\beta_1^+$ cells present (Yun *et al.*, 2000). Cells expressing CD4, CD8α, TCRγδ, $TCR\alpha\beta_1$ or $TCR\alpha\beta_2$ markers are capable of producing IFN-γ and any or all of these cells (with the exception of $TCR\alpha\beta_2^+$ cells) could be at least partially responsible for the

increase in *IFNG* mRNA that occurs following *Eimeria* infection. At no point were TCRαβ$_2^+$ cells observed in the gut. Previously, TCRαβ$_2^+$ cells were found to be rarely located within the gut of chickens but on the rare occasion when they were present they were localised to the lamina propria (Char *et al.*, 1990).

Macrophages and CD4$^+$ cells are the main producers of IL-10, however B cells, CD8$^+$ cells and innate cells other than macrophages are also capable of IL-10 production. IL-10 is a defining cytokine of Tregs (CD4$^+$CD25$^+$) in mammals and chickens (Shanmugasundaram *et al.*, 2011). The fact that large clusters of CD4$^+$ cells were observed following *E. maxima* infection but, surprisingly, no changes are observed to the CD25$^+$ cell population, suggests that CD4$^+$CD25$^+$ Tregs are not the main producers of *IL10* mRNA observed here. In contrast to this, *E. tenella* infection did induce increases to the CD25$^+$ population in the caecum, although not to the extent that CD4$^+$ cells were increased which may indicate that some CD4$^+$CD25$^+$ cells are involved in the response to *E. tenella*. However, IL-10 was only significantly increased at 8 dpi, whereas increased numbers of CD4$^+$ cells were observed from 3 dpi, therefore Tregs are unlikely the producers of IL-10 during *E. tenella* infection. CD25 is also an activation marker and the increases in CD4$^+$ and CD25$^+$ cells could indicate an increase in activated T cells following *E. tenella* infection. The staining patterns of CD25$^+$ IELs revealed by histology resembled that of CD8α$^+$ cells but the pattern of CD25$^+$ LPLs was similar to both CD8α$^+$ and CD4$^+$ cells. *E. maxima* and *E. tenella* elicit different pathologies; *E. maxima* causes malabsorption whereas *E. tenella* is haemorrhagic in its nature. Another possibility is that a dosage of 2 x10^3 *E. maxima* oocysts is not enough to cause sufficient pathology to elicit a strong Treg response.

IL-10 is most commonly considered a regulatory cytokine in both chickens and mammals, which functions to dampen Th1 and Th17 (Chaudhry *et al.*, 2011) responses by suppressing the production of proinflammatory mediators such as IFN-γ (Rothwell *et al.*, 2004). However, a number of other functions of IL-10 have been found in mammals, including but not limited to wound healing and repair (Peranteau *et al.*, 2008), gut homeostasis (Kuhn *et al.*, 1993), promoting maturation of memory $CD8^+$ T cells (Laidlaw *et al.*, 2015) regulating immune tolerance (Shouval *et al.*, 2014) and enhancing NK cell cytotoxicity (Mocellin *et al.*, 2004). However the sources of IL-10 and the functions it has during *Eimeria* infection are still to be determined. It is possible that IL-10 is produced as either an anti-inflammatory mediator to dampen the immune response and limit immune induced-pathology during *Eimeria* infection or to facilitate healing and tissue repair following infection. Here, given the positive correlation between *IL10* mRNA and parasite genome copy number, it seems likely that IL-10 is either responsible for controlling the inflammatory response induced by *Eimeria* infection, in particular *E. maxima*, or is induced by the parasite itself to dampen the inflammatory response and limit its efficacy against *Eimeria.* In a study by Rothwell *et al.* (2004) the *E. maxima*-susceptible chicken line 15I had higher *IL10* mRNA in the jejunum following infection than that of *E. maxima*-resistant chicken line C.B12, indicating that IL-10 reduces the efficacy of the immune response to *E. maxima*. IL-10 produced at early time points (4 dpi for *E. maxima* and for *E. tenella*, 5 dpi, although this was not significant) may be important to controlling the innate inflammatory response whereas IL-10 produced later on (8 dpi onwards for *E. maxima* and 8 dpi for *E. tenella*) may be essential to the repair of tissue damage caused by infection. During

the immune response to *T. gondii,* IL-10 is vital to the control of immunopathology. IL-$10^{-/-}$ mice acutely infected with *T. gondii* demonstrated lethal immunopathology due to uncontrolled production of TNF-α and IFN-γ (Gazzinelli *et al.*, 1996).

The proportion of macrophages (expressing MRC1L-B) did not change dramatically over the course of infection with either *E. maxima* or *E. tenella*. In contrast with this, a previous study showed that numbers of macrophages (recognised by CVI-ChNL-68.1 marker) increased in *E. tenella*-naïve birds given an intra-caecal inoculation of *E. tenella* sporozoites (Vervelde *et al.*, 1996). The unchanging numbers of macrophages observed here during *Eimeria* infection does not rule these cells out as a source of IL-10 as it is likely that the activation state of the macrophages changes once exposed to *Eimeria* antigens. No discernible changes were observed to the $Tim4^{+}$ cell population throughout this experiment.

Previously, Rose *et al.* (1979) found that bursectomised (B cell deficient) chickens had increased oocyst production after primary infection with *E. maxima* and were less resistant to secondary infection than un-bursectomised birds, although substantial immunity was still observed. Here, clusters of $chB6^{+}$ cells were observed in the lamina propria following both *E. maxima* and *E. tenella* infection which indicates that B cells are involved in the response to *Eimeria.*

Previous studies in mice have shown NK cells to not be important to immunity to *Eimeria vermiformis* (Smith *et al.*, 1994; Rose *et al.*, 1995). However, an increase in intraepithelial NK cell cytotoxic activity was observed at 8 dpi in chickens following *E. acervulina* infection but no NK cell activity was observed in lamina propria cells following infection (Lillehoj, 1989). The presence and increased numbers following

Eimeria infection of chB6$^+$ and CD8α$^+$ IELs in the jejunum and caecum observed in this trial may indicate increased NK cell activity, although the lack of corresponding CD25$^+$ cells and different staining patterns observed between chB6$^+$ and CD25$^+$ cells deems this unlikely. Therefore, the clusters of chB6$^+$ cells in the lamina propria following both *E. maxima* and *E. tenella* are presumably B cells.

The majority of cell clusters observed were localised to the lamina propria. It is likely that immune cells are migrating to areas in the tissues where the *Eimeria* is located. However, attempts to stain tissues with *Eimeria*-specific antibodies were unsuccessful therefore this cannot be confirmed.

The main finding of this experiment is that Th17 cells are not involved during *Eimeria* infection in Ross 308 broilers. However, IL-21 was identified as having a novel role in the response to both *E. maxima* and *E. tenella* and *IL10* was increased following infection. Both IL-10 and IL-21 are multi-functional and further investigation would be required to define the roles of these cytokines in response to *Eimeria* infection, and to identify which cells are the major producers of IL-10 and IL-21 during infection.

Chapter 5 Phenotypes and biomarkers of disease resistance and susceptibility to *E. maxima* and *E. tenella*.

5.1 Introduction

Previous studies have shown that different lines of chickens exhibit varying degrees of susceptibility and resistance to different *Eimeria* species. Two of these lines, 15I and C.B12 exhibit inverse resistance and susceptibility to *E. maxima* and *E. tenella.* The C.B12 and 15I White Leghorn chicken lines were bred for MHC haplotypes B12 and B15 respectively. Line C.B12 chickens are considered *E. maxima*-resistant and *E. tenella*-susceptible whereas line 15I chickens are considered *E. maxima*-susceptible and *E. tenella*-resistant (Bumstead *et al.*, 1995; Smith *et al.*, 2002). Following primary infection with *E. maxima,* C.B12 chickens produce fewer oocysts than 15I chickens however both lines develop complete protection to secondary infection and no oocysts are produced after secondary challenge (Bumstead *et al.*, 1995; Smith *et al.*, 2002). On primary infection with *E. tenella,* both lines produce similar numbers of oocysts, however line 15I chickens develop a level of protective immunity and produce fewer oocysts than line C.B12 chickens after secondary infection (Bumstead *et al.*, 1995). Very little is known about the immune mechanisms underpinning resistance and susceptibility to *E. maxima* and *E. tenella* in these two lines. Rothwell *et al.* (2004) observed higher *IL10* mRNA expression in the small intestine of *E. maxima*-infected line 15I chickens compared with line C.B12 at 6 dpi, indicating that higher IL-10 may be detrimental to the immune response to *Eimeria* and clearance of the pathogen.

The hypothesis to be tested is that two inbred chicken lines which differ in their resistance and susceptibility to *E. maxima* and *E. tenella,* exhibit differential immune responses upon infection with both these *Eimeria* species. The main aim of this chapter is to identify phenotypes and biomarkers of *Eimeria* resistance. In line

C.B12 and 15I chickens infected with *E. maxima* and *E. tenella*, changes to cytokine expression in the gut were measured by RT-qPCR. To further characterise the role of IL-10 and IFNγ in resistance to *Eimeria*, IL-10 and IFNγ in the serum and IL-10 in the gut tissue of these lines were also measured by ELISA. Changes to cell populations in the gut during infection were analysed by ICC to determine which cell types are important in producing IL-10, IFNγ and other key cytokines during infection.

5.2 Results

5.2.1 Percentage weight gains in lines 15I and C.B12 following *E. maxima* and *E. tenella* infection.

The percentage weight gain for each bird was calculated from the weights of the birds from two days prior to inoculation to the time of culling. Throughout infection with *E. maxima* (Figure 5-1A) or *E. tenella* (Figure 5-1B) there were no significant changes to percentage weight gains between the two lines or between infected and control birds.

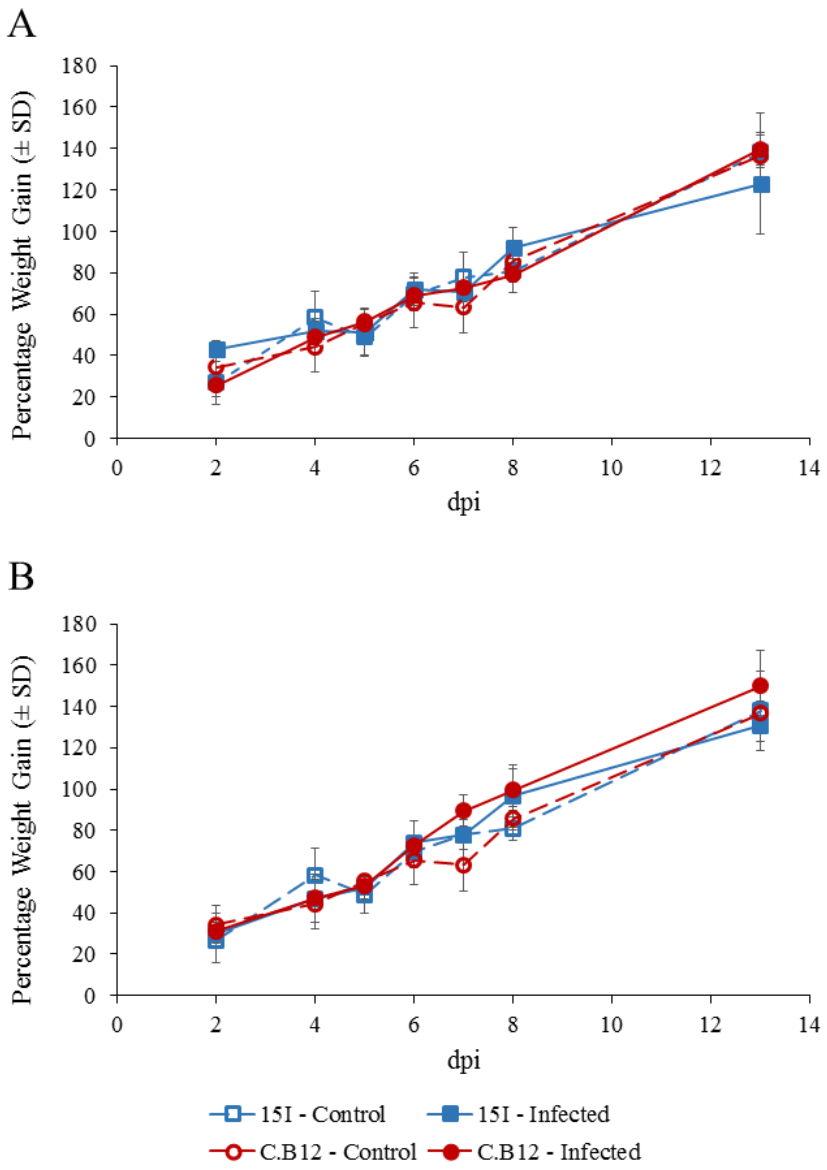

Figure 5-1: Percentage weight gains in line 15I and C.B12 chickens following *E. maxima* (A) or *E. tenella* (B) infection. Three-week-old birds were orally inoculated with 100 *E. maxima* oocysts, 200 *E. tenella* oocysts or sterile water (control birds). Percentage weight gain was calculated for individual birds from 2 days prior to inoculation to time of culling. Shown is the mean percentage weight gain ± SD for each line at each time point; n=3 for control and n=5 or more for infected groups.

5.2.2 *E. maxima* and *E. tenella* replication in the gut of infected 15I and C.B12 chickens.

E. maxima and *E. tenella* genomic DNA in infected tissues were quantified by two qPCRs, developed for species-specific detection, by Dr Matt Nolan (RVC, UK). Following *E. maxima* infection, susceptible line 15I birds had significantly ($p<0.05$) higher *E. maxima* genome copy numbers in the jejunum than line C.B12 birds (Figure 5-2A).

In all *E. tenella*-infected birds at 2 dpi, five of six of line 15I at 4 dpi and two of five of line C.B12 at 4 dpi, the qPCR returned results with high C*t* values (>33) and the melting curves of the DNA indicated that it was not *E. tenella* DNA that was being amplified, therefore these birds were not included in the analysis. Following *E. tenella* infection, there was no significant difference in parasite copy numbers between the two lines at any time point (Figure 5-2B).

To check that control birds were negative for *Eimeria* DNA, cDNA was produced from the RNA extracted for RT-qPCR and PCR was ran for detection of the *Eimeria* 5S ribosomal RNA (rRNA) gene (Blake *et al.*, 2006). *Eimeria* 5S rRNA was not detected in samples from the control birds of either line at any time point indicating that control birds were free from *Eimeria*.

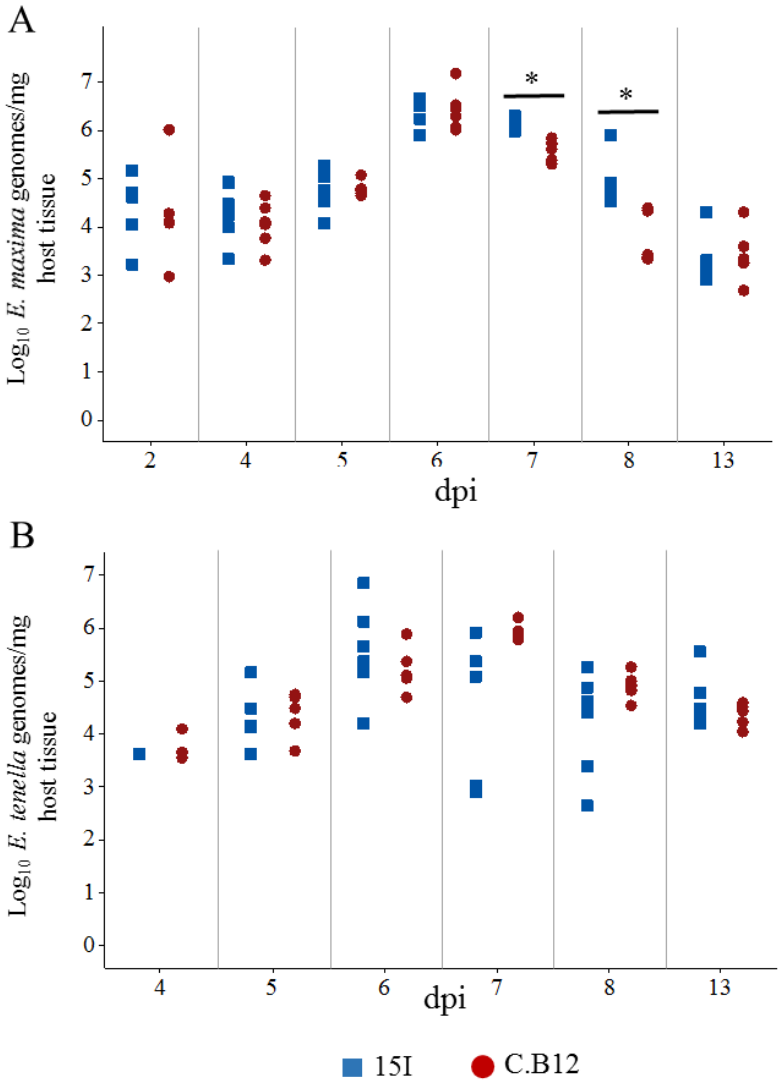

Figure 5-2: Parasite replication in the jejunum of *E. maxima*- and caecum of *E. tenella*-infected line 15I and C.B12 chickens as determined by qPCR. Three-week-old birds were orally inoculated with 100 oocysts of *E. maxima* (A) or 200 oocysts of *E. tenella* (B) and tissues collected at various time points post infection. *E. maxima* was quantified by an established qPCR method targeting the *MIC1* gene (Blake *et al.*, 2006). A 10 cm length of jejunum (spanning 5 cm either side of the MD) was collected for total genomic DNA extraction. *E. tenella* was quantified by an established qPCR method targeting the RAPD-SCAR marker Tn-E03-1161 (Nolan *et al.*, 2015). Residual caeca were pooled within birds for total genomic DNA extraction. Shown are the $\log_{10}$ transformed parasite genome copy numbers per mg of tissue for individual birds. Asterisks indicate significant differences between the two lines at $p<0.05$, Mann-Whitney U Test; $n\geq5$ for each time point with the exception of *E. tenella* infected birds at 4 dpi where n=1 for line 15I and n=3 for line C.B12 birds.

5.2.3 Changes to cytokine expression in the gut of *E. maxima* and *E. tenella* infected 15I and C.B12 chickens.

RNA was extracted from tissues and changes to the mRNA levels of cytokines were quantified by TaqMan® RT-qPCR as in Section 2.2.2. To check whether a particular gene was altered during infection, RNA samples were first pooled within each group at each time point before RT-qPCR analysis. Two µg of total RNA from each sample at 2, 6 and 13 dpi was pooled and total volumes made up to 20 µl with RNase-free water. If the expression of genes changed following infection, expression of that gene was measured in individual birds.

As IL-10 is thought to be a biomarker for susceptibility to *Eimeria, IL10* mRNA expression was quantified by RT-qPCR in the jejunum of control and *E. maxima*-infected birds (Figure 5-3A) and the mid-caecum of control and *E. tenella*-infected birds (Figure 5-3B). In this chapter, the primers and probe set used to quantify *IL10* mRNA was IL10 (2) as given in Table 2-1 (Chapter 2). Following *E. maxima* infection, *IL10* increased in both lines. At early time points, *IL10* expression in the jejunum was significantly ($p<0.05$) higher in *E. maxima*-infected resistant line C.B12 chickens than in line 15I chickens however at later time points, signficantly ($p<0.05$) higher *IL10* mRNA was observed in infected susceptible line 15I birds than in line C.B12 birds.

Following *E. tenella* infection, *IL10* mRNA expression was increased in both lines but overall, there was no major difference in *IL10* mRNA expression in the caecum of the two lines, in either *E. tenella*-infected or uninfected birds.

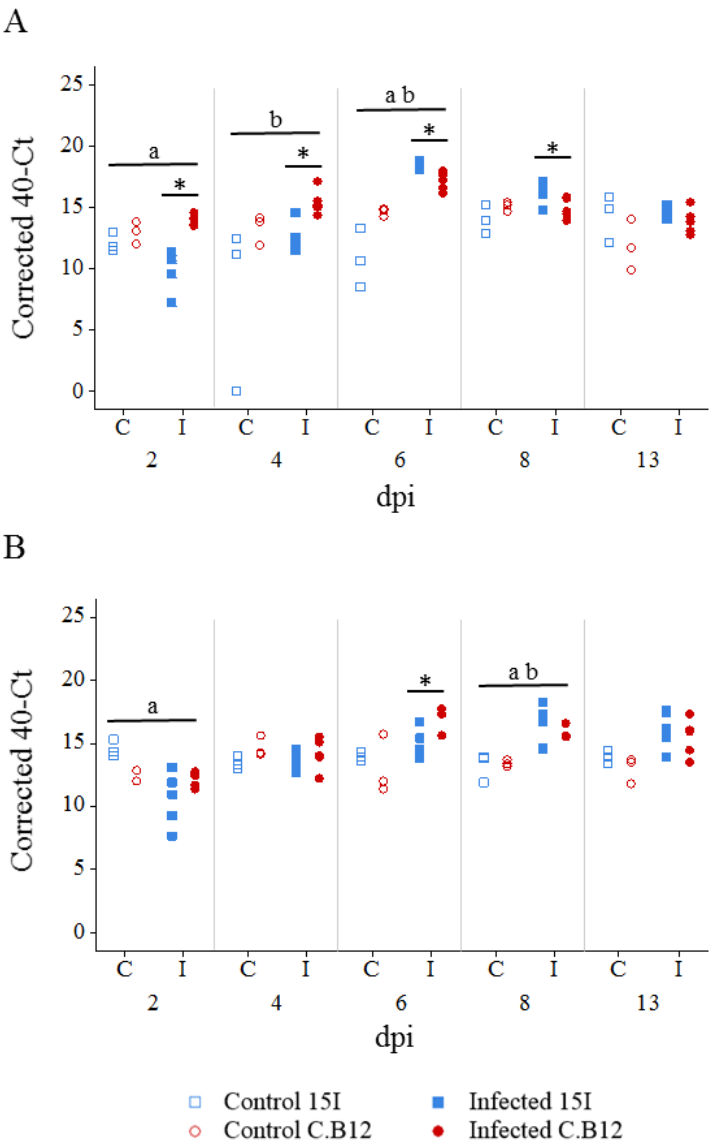

Figure 5-3: *IL10* mRNA expression in the jejunum of *E. maxima*-infected (A) and caecum of *E. tenella*-infected (B) line 15I and C.B12 chickens. Three-week-old birds were orally inoculated with 100 oocysts of *E. maxima*, 200 oocysts of *E. tenella* or sterile water (control birds) and samples collected at various time points post infection. Data are presented as individual birds. Significant differences between the two lines given the same treatment are indicated by an asterisk, between 15I control and infected birds by an 'a' and between C.B12 control and infected birds by a 'b' ($p<0.05$, Mann-Whitney U Test); n=3 for control groups and n=5 or more for infected groups. C; Control. I; Infected.

As IFNγ is important during the immune response to and for clearance of the *Eimeria* parasite, *IFNG* expression was measured. *IFNG* mRNA was increased in the pooled sample set and was therefore measured in individual birds. Following *E. maxima* infection (Figure 5-4A), *IFNG* was significantly ($p<0.05$) upregulated in the jejunum of both lines in comparison with control birds. A similar pattern of *IFNG* expression was observed in the birds as for *IL10* expression. At early time points, *IFNG* expression was higher in infected resistant line C.B12 birds than line 15I but during the course of infection this changed and *IFNG* expression was higher in infected line 15I birds compared with C.B12.

Following *E. tenella* infection (Figure 5-4B), *IFNG* mRNA expression was increased in both lines. Overall, there was no major difference in *IFNG* mRNA expression in the caecum of the two lines, in either infected or uninfected birds.

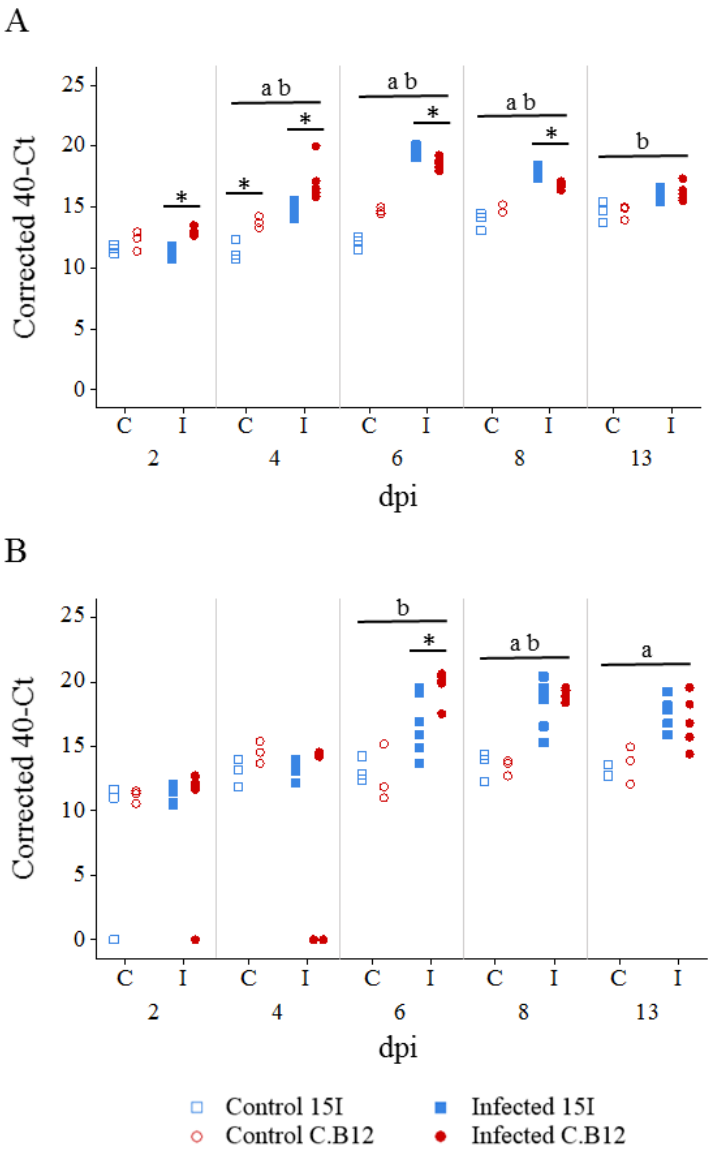

Figure 5-4: *IFNG* mRNA expression in the jejunum of *E. maxima*-infected (A) and caecum of *E. tenella*-infected (B) line 15I and C.B12 chickens. Three-week-old birds were orally inoculated with 100 oocysts of *E. maxima*, 200 oocysts of *E. tenella* or sterile water (control birds) and samples collected at various time points post infection. Data are presented as individual birds. Significant differences between the two lines given the same treatment are indicated by an asterisk, between 15I control and infected birds by an 'a' and between C.B12 control and infected birds by a 'b' ($p<0.05$, Mann-Whitney U Test); n=3 for control groups and n=5 or more for infected groups. C; Control. I; Infected.

To check for potential differences in Th2 responses between the two lines during *Eimeria* infection, *IL13* expression was measured in the pooled RNA sample set (Figure 5-5). No changes to *IL13* expression were detected, either in response to *E. maxima* or *E. tenella* infection or between the two chicken lines and so *IL13* was not measured in individual samples.

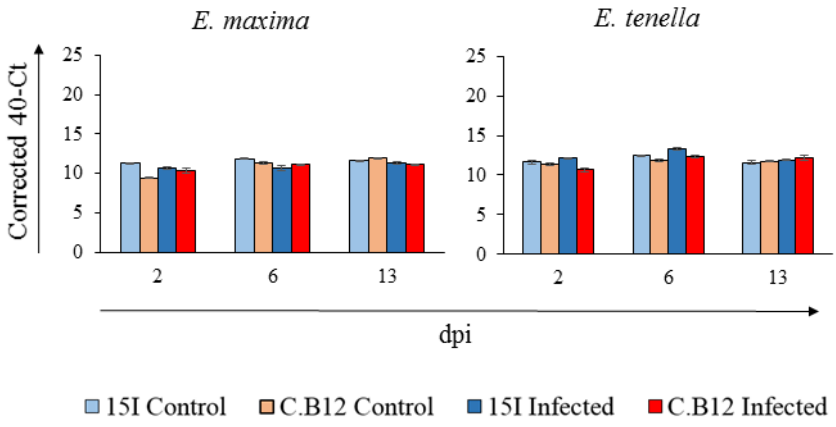

Figure 5-5: *IL13* mRNA expression in the jejunum of *E. maxima*-infected and caecum of *E. tenella*-infected line 15I and C.B12 chickens. Three-week-old birds were orally inoculated with 100 oocysts of *E. maxima*, 200 oocysts of *E. tenella* or sterile water (control birds) and samples collected at various time points post infection. Equal quantities of RNA were pooled within birds of the same line and treatment group at each time point (n=3 for control and n=5 for infected birds) and *IL13* expression measured by RT-qPCR. Data are presented as the mean corrected 40-Ct values for triplicate wells ± SEM.

IL17A and *IL17F* expression was measured in the pooled RNA sample set. No major changes were observed in *IL17A* (Figure 5-6A) or *IL17F* (Figure 5-6B) expression following *Eimeria* infection or between the two lines and so these cytokines were not measured in samples from individual birds.

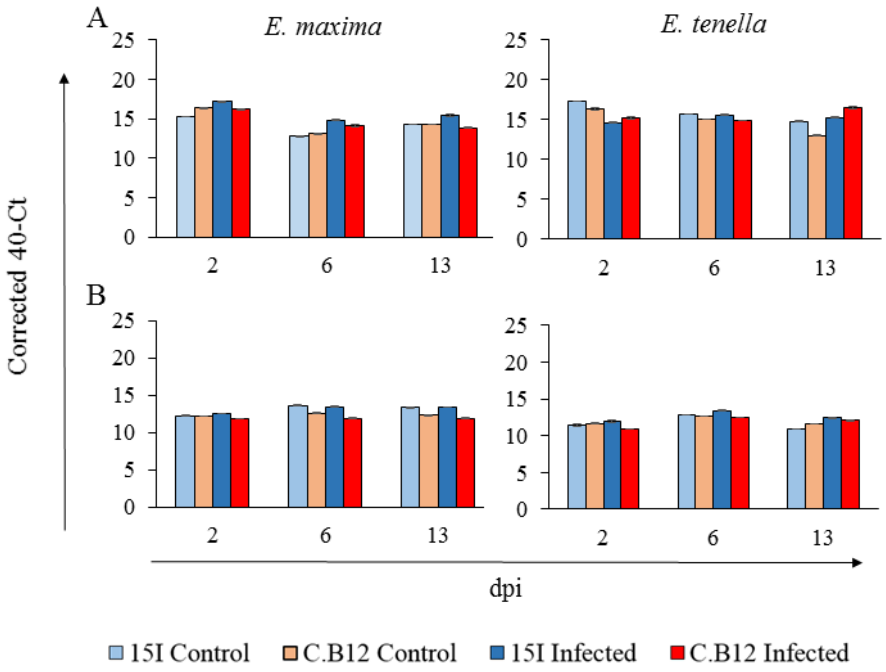

Figure 5-6: *IL17A* (A) and *IL17F* (B) mRNA expression in the jejunum of *E. maxima*-infected and caecum of *E. tenella*-infected line 15I and C.B12 chickens. Three-week-old birds were orally inoculated with 100 oocysts of *E. maxima*, 200 oocysts of *E. tenella* or sterile water (control birds) and samples collected at various time points post infection. Equal quantities of RNA were pooled within birds of the same line and treatment group at each time point (n=3 for control and n=5 for infected birds) and *IL17A* and *IL17F* expression measured by RT-qPCR. Data are presented as the mean corrected 40-Ct values for triplicate wells ± SEM.

IL21 expression was measured from the pooled sample set and was changed following both *E. maxima* and *E. tenella* infection and so was measured in individual samples (Figure 5-7). Following *E. maxima* infection, *IL21* expression increased significantly in the jejunum of infected susceptible line 15I birds compared to control 15I birds but did not increase in infected line C.B12 birds compared to control C.B12 birds (Figure 5-7A). Due to the high variation within control groups, statistical analysis was repeated against line 15I control birds from all time points to determine if the differences observed were a true result of infection or due to natural variation between the birds. Only the increased *IL21* in line 15I at 6 dpi was found to be due to infection and so the increase observed at 4 dpi must be interpreted with caution. *IL21* expression during *E. maxima* infection followed a similar pattern as *IFNG* and *IL10* expression. At early time points, infected resistant line C.B12 birds had higher *IL21* mRNA levels than infected 15I birds, but, as with *IL10* and *IFNG* expression this was reversed at later time points and infected susceptible line 15I birds expressed more *IL21* mRNA than infected C.B12 birds.

Following *E. tenella* infection, *IL21* was increased in infected birds of both lines at later time points compared to control birds (Figure 5-7B). No differences in *IL21* expression were observed between the two lines in either control or *E. tenella*-infected birds. In both the jejunum and caecum there were several birds of each line which failed to produce *IL21*.

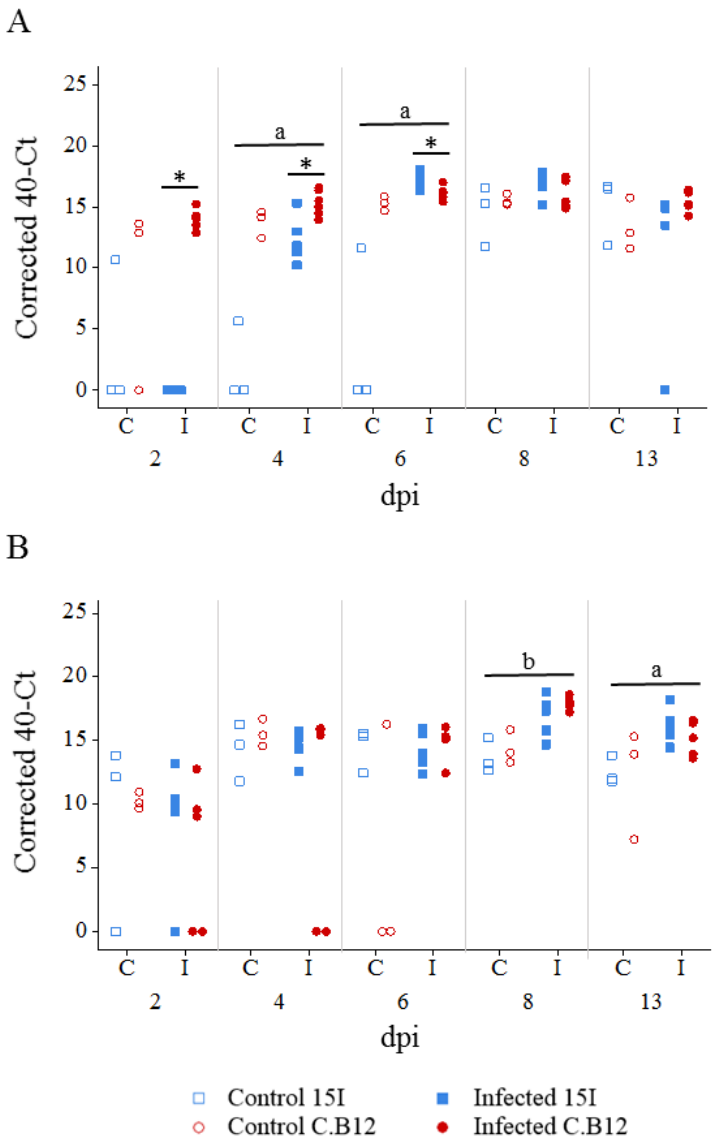

Figure 5-7: *IL21* mRNA expression in the jejunum of *E. maxima*-infected (A) and caecum of *E. tenella*-infected (B) line 15I and C.B12 chickens. Three-week-old birds were orally inoculated with 100 oocysts of *E. maxima*, 200 oocysts of *E. tenella* or sterile water (control birds) and samples collected at various time points post infection. Data are presented as individual birds. Significant differences between the two lines given the same treatment are indicated by an asterisk, between 15I control and infected birds by an 'a' and between C.B12 control and infected birds by a 'b' ($p<0.05$, Mann-Whitney U Test); n=3 for control groups and n=5 or more for infected groups. C; Control. I; Infected.

IL2 expression was measured in the pooled RNA sample set and appeared to be upregulated following *Eimeria* infection and was therefore measured in individual samples (Figure 5-8). Overall *IL2* expression did not change following infection in either line when measured in individual birds.

No significant differences were observed in *IL2* expression between the two chicken lines after *E. tenella* infection. However, *IL2* expression did increase significantly ($p<0.05$) in both lines at later time points, compared with control birds.

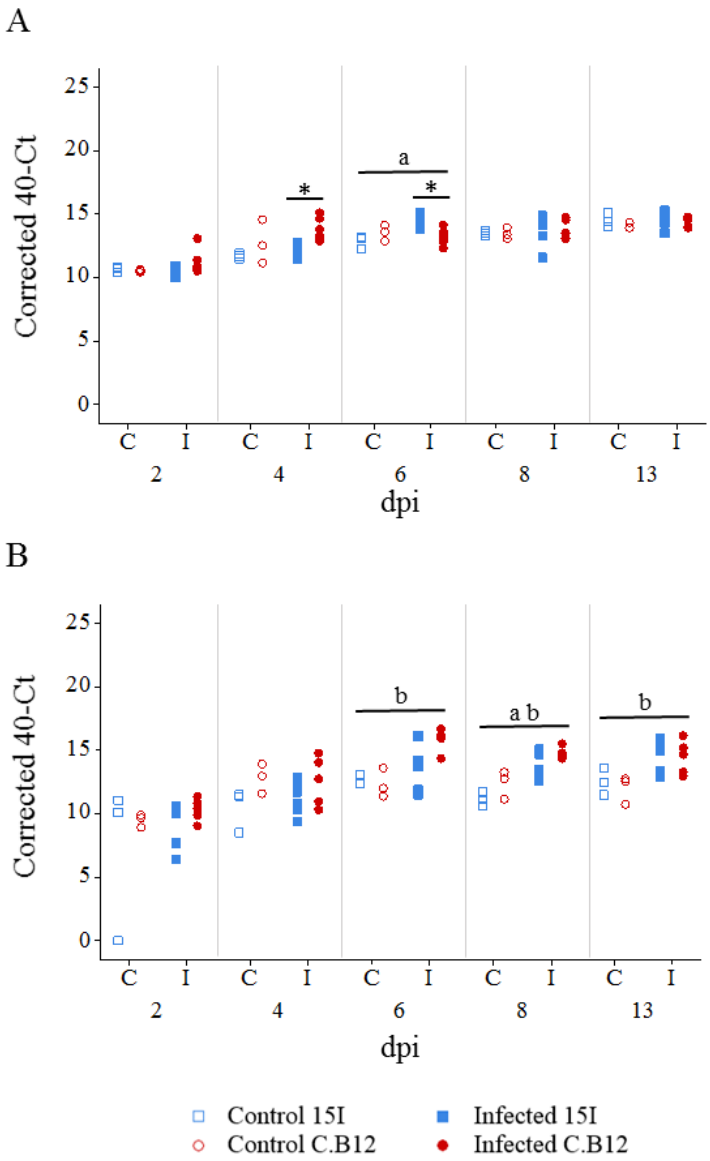

Figure 5-8: *IL2* mRNA expression in the jejunum of *E. maxima*-infected (A) and caecum of *E. tenella*-infected (B) line 15I and C.B12 chickens. Three-week-old birds were orally inoculated with 100 oocysts of *E. maxima*, 200 oocysts of *E. tenella* or sterile water (control birds) and samples collected at various time points post infection. Data are presented as individual birds. Significant differences between the two lines given the same treatment are indicated by an asterisk, between 15I control and infected birds by an 'a' and between C.B12 control and infected birds by a 'b' ($p<0.05$, Mann-Whitney U Test); n=3 for control groups and n=5 or more for infected groups. C; Control. I; Infected.

IL6 mRNA was measured as it has previously been associated with resistance to *E. vermiformis* in mice (Lynagh *et al.*, 2000). *IL6* appeared to be changed in response to infection in the pooled sample set and so was measured in individual samples (Figure 5-9). Overall, *IL6* mRNA expression was unchanged in *E. maxima*-infected compared to control birds and no differences were observed between the two lines. Overall, no changes in *IL6* expression were observed as a result of infection or in the caecum between the two lines following *E. tenella* infection.

Due to the high variation in *IL6* expression in both control and infected birds, significant results were retested against the data from all control birds of that line from each time point. On both occasions the increases originally observed were not significant when compared to all control birds of that line indicating that the initial significance observed was a result of natural variation between individual birds rather than an effect of *Eimeria* infection.

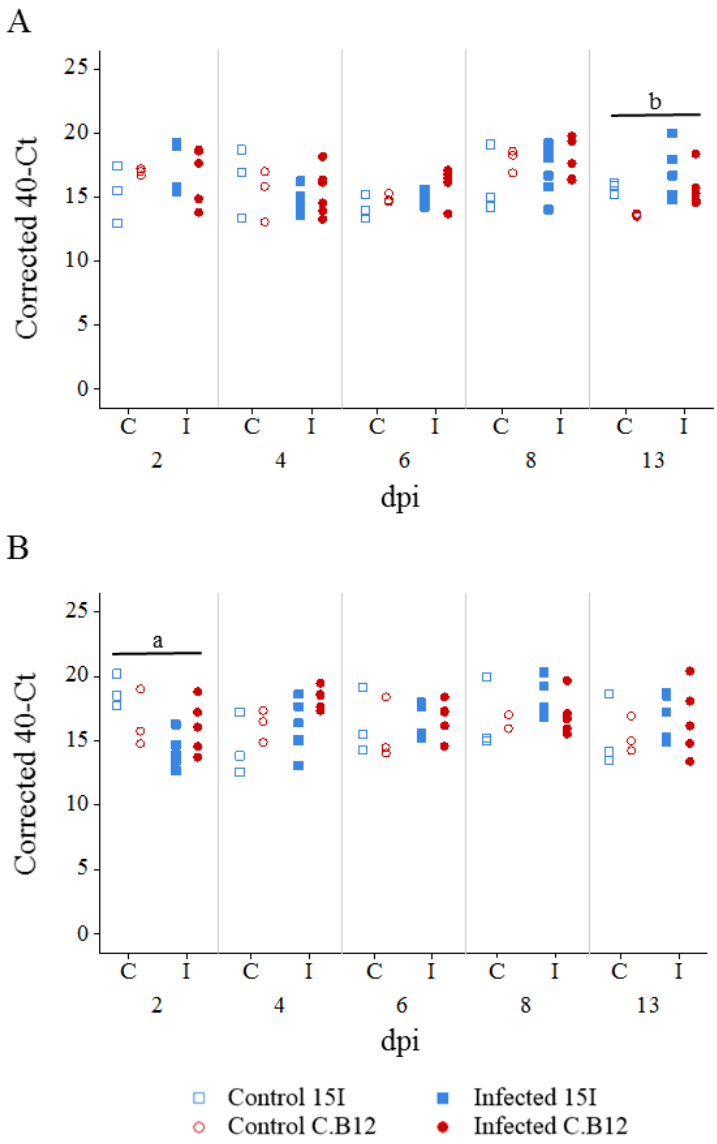

Figure 5-9: *IL6* mRNA expression in the jejunum of *E. maxima*-infected (A) and caecum of *E. tenella*-infected (B) line 15I and C.B12 chickens. Three-week-old birds were orally inoculated with 100 oocysts of *E. maxima*, 200 oocysts of *E. tenella* or sterile water (control birds) and samples collected at various time points post infection. Data are presented as individual birds. Significant differences between the two lines given the same treatment are indicated by an asterisk, between 15I control and infected birds by an 'a' and between C.B12 control and infected birds by a 'b' ($p<0.05$, Mann-Whitney U Test); n=3 for control groups and n=5 or more for infected groups. C; Control. I; Infected.

To examine the relationship between the expression of *IL10*, *IFNG*, *IL21*, *IL2* and *IL6* and parasite burden in the gut during *Eimeria* infection, the correlation between the expression of these cytokines and *Eimeria* genome copy number per mg of host tissue was calculated (Table 5-1). *E. maxima* genome copy number was found to correlate positively with *IL10, IFNG* and *IL21* expression in both lines. *E. tenella* genome copy number correlated positively with *IL10*, *IFNG*, *IL21* and *IL2* expression in the caecum of both lines and for *IL6* expression in line 15I birds only.

Table 5-1*:* Spearman's rank correlation coefficients between *IL10*, *IFNG*, *IL21*, *IL2* and *IL6* mRNA expression and *E. maxima* (n=41 for line 15I and n=39 for line C.B12) and *E. tenella* (n=31 for line 15I and n=29 for line C.B12) genome copy number in the jejunum and caecum of infected birds. Shown are the Spearman's correlation coefficients (ρ) for data of all infected birds pooled from all time points, calculated between the mean corrected 40-Ct of triplicate wells of two cytokines in individual birds. P values were calculated using the two-tailed t test and were considered significant at $p<0.05$ and highly significant at $p<0.001$.

	E. maxima		*E. tenella*	
	15I	C.B12	15I	C.B12
IL10	0.58 ($p<0.05$)	0.68 ($p<0.001$)	0.75 ($p<0.001$)	0.81 ($p<0.001$)
IFNG	0.51 ($p<0.05$)	0.55 ($p<0.05$)	0.81 ($p<0.001$)	0.86 ($p<0.001$)
IL21	0.56 ($p<0.05$)	0.18 ($p=0.37$)	0.51 ($p<0.05$)	0.58 ($p<0.05$)
IL2	0.19 ($p=0.35$)	-0.23 ($p=0.26$)	0.83 ($p<0.001$)	0.75 ($p<0.001$)
IL6	-0.11 ($p=0.59$)	-0.03 ($p=0.90$)	0.51 ($p<0.05$)	-0.10 ($p=0.61$)

As similar patterns of expression were observed for *IL10*, *IFNG* and *IL21* following *E. maxima* infection, the relationship between the levels of cytokine expression was examined (Table 5-2). *IL10*, *IFNG*, *IL21, IL2* and *IL6* expression in the jejunum all correlated positively with one another following infection with *E. maxima*, however the correlation between *IL6* and the other cytokines was not as strong.

Table 5-2: Spearman's rank correlation coefficients between *IL10*, *IFNG*, *IL21*, *IL2* and *IL6* mRNA expression in the jejunum following *E. maxima* infection. Shown are the Spearman's correlation coefficients (ρ) for data of all birds pooled from all time points, calculated between the mean corrected 40-Ct of triplicate wells of two cytokines in individual birds. P values were calculated using the two-tailed t test and were considered significant at $p<0.05$ and highly significant at $p<0.001$; n=85.

	IL10	*IFNG*	*IL21*	*IL2*	*IL6*
IL10		0.85 (p<0.001)	0.86 (p<0.001)	0.59 (p<0.001)	0.20 (p=0.07)
IFNG	0.85 (p<0.001)		0.80 (p<0.001)	0.67 (p<0.001)	0.03 (p=0.81)
IL21	0.86 (p<0.001)	0.80 (p<0.001)		0.66 (p<0.001)	0.13 (p=0.23)
IL2	0.59 (p<0.001)	0.67 (p<0.001)	0.66 (p<0.001)		0.00 (p=0.97)
IL6	0.20 (p=0.07)	0.03 (p=0.81)	0.13 (p=0.23)	0.00 (p=0.97)	

The relationship between the expression of *IL10*, *IFNG*, *IL21*, *IL2* and *IL6* in the caecum of *E. tenella* infected birds was also examined (Table 5-3). *IL10*, *IFNG*, *IL21*, *IL2* and *IL6* expression all correlated positively with one another, but as in the case with *E. maxima*, the correlation between *IL6* expression and the expression of the other cytokines was not as strong.

Table 5-3: Spearman's rank correlation coefficients between *IL10*, *IFNG*, *IL21*, *IL2* and *IL6* mRNA expression in the caecum following *E. tenella* infection. Shown are the Spearman's correlation coefficients (ρ) for data of all birds pooled from all time points, calculated between the mean corrected 40-Ct of triplicate wells for two cytokines in individual birds. P values were calculated using the two-tailed t test and were considered significant at $p<0.05$ and highly significant at $p<0.001$; n=85.

	IL10	*IFNG*	*IL21*	*IL2*	*IL6*
IL10		0.86 (p<0.001)	0.76 (p<0.001)	0.83 (p<0.001)	0.28 (p<0.05)
IFNG	0.86 (p<0.001)		0.82 (p<0.001)	0.89 (p<0.001)	0.11 (p=0.31)
IL21	0.76 (p<0.001)	0.82 (p<0.001)		0.73 (p<0.001)	0.12 (p=0.26)
IL2	0.83 (p<0.001)	0.89 (p<0.001)	0.73 (p<0.001)		0.13 (p=0.23)
IL6	0.28 (p<0.05)	0.11 (p=0.31)	0.12 (p=0.26)	0.13 (p=0.23)	

5.2.4 IL-10 concentration in gut tissue of *E. maxima*- and *E. tenella*-infected 15I and C.B12 chickens.

The concentration of IL-10 in homogenised jejunum and caecum tissues was quantified by Dr Sungwon Kim (Roslin Institute, UK) by ELISA (Wu *et al.*, 2016). Tissue IL-10 increased in the jejunum of both lines after *E. maxima* infection. The level of IL-10 in the jejunum tissue of resistant line C.B12 birds infected with *E. maxima* was initially higher than that of infected 15I birds, however at later time points, this was reversed and line 15I birds had higher levels of IL-10 than infected C.B12 birds, although these differences were not significant (Figure 5-10A).

IL-10 in the caecum of both lines increased after *E. tenella* infection, but only significantly ($p<0.05$) in susceptible line C.B12 and overall no difference was observed between the two lines (Figure 5-10B).

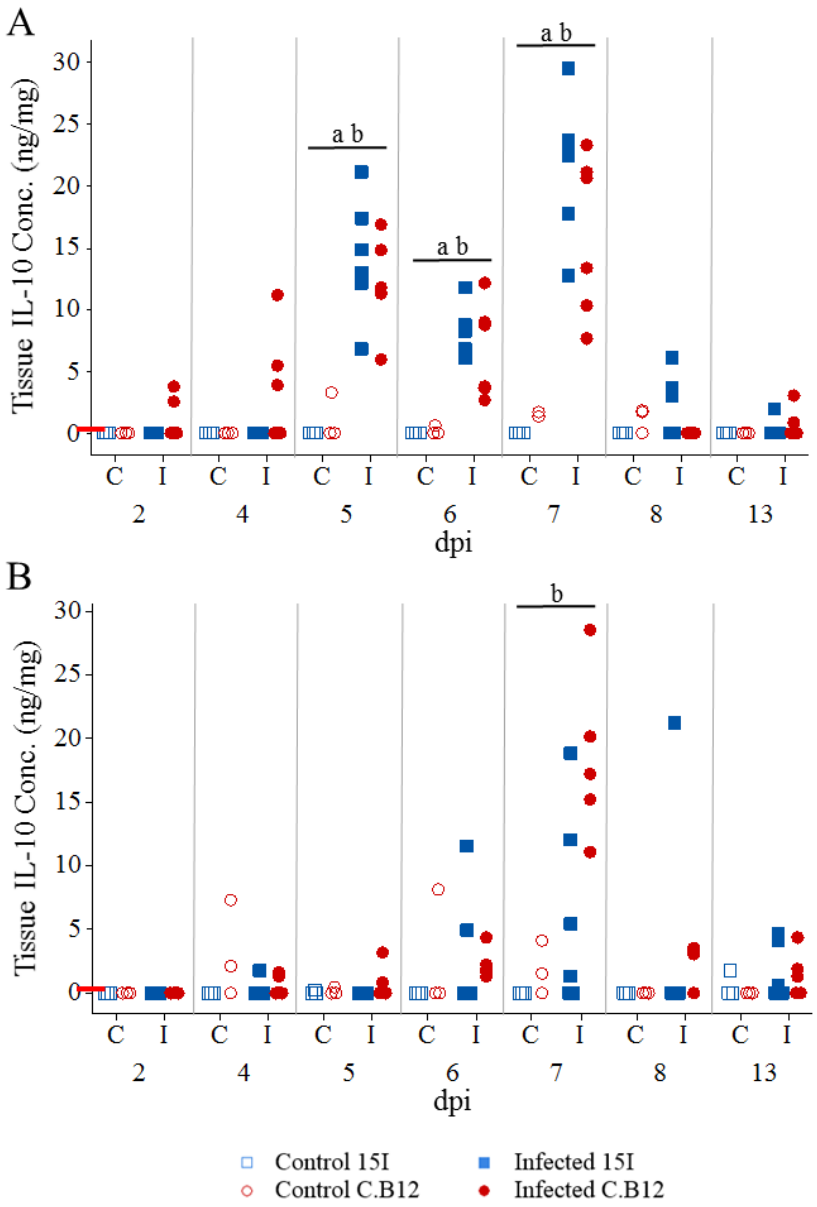

Figure 5-10: IL-10 in the jejunum and caecum of line 15I and C.B12 chickens infected with *E. maxima* (A) or *E. tenella* (B) respectively. Three-week-old birds were orally inoculated with 100 *E. maxima* oocysts, 200 *E. tenella* oocysts or sterile water (control birds) and jejunum or caecum tissue collected and snap-frozen at culling at various time points post infection. Tissues were homogenised and the concentration of IL-10 quantified by ELISA. Data are presented as individual birds. Red tick mark on the *y* axis indicates the detection threshold of the assay (30 pg/ml). Significant differences between the two lines given the same treatment are indicated by an asterisk, between 15I control and infected birds by an 'a' and between C.B12 control and infected birds by a 'b' ($p<0.05$, Mann-Whitney U Test); n=3 for control groups and n=5 or more for infected groups.

To examine the relationship between protein IL-10 in gut tissue and *Eimeria* genome copy number, the Spearman's rank correlation coefficient was correlated between the two. The concentration of IL-10 in gut homogenate positively correlated with *E. maxima* and *E. tenella* genome copy numbers in the jejunum and caecum respectively of both lines (Table 5-4).

Table 5-4: Spearman's rank correlation coefficients between the concentration of IL-10 and the *Eimeria* genome copy number in the jejunum following *E. maxima* infection (n=41 for line 15I and n=39 for line C.B12) and caecum following *E. tenella* infection (n=31 for line 15I and n=29 for line C.B12). Shown are the Spearman's correlation coefficient (ρ) for data of all infected birds pooled within lines from all time points. P values were calculated using the two-tailed t test and were considered significant at $p<0.05$ and highly significant at $p<0.001$.

E. maxima		*E. tenella*	
15I	C.B12	15I	C.B12
0.64 ($p<0.001$)	0.57 ($p<0.001$)	0.40 ($p=0.032$)	0.81 ($p<0.001$)

5.2.5 Serum IL-10 in *E. maxima*- and *E. tenella*-infected 15I and C.B12 chickens.

Serum IL-10 was quantified in control and infected birds of both lines by ELISA as by Wu *et al.* (2016). IL-10 was significantly ($p<0.05$) increased in the serum of both lines in response to *E. maxima* infection. The level of IL-10 observed in resistant line C.B12 birds infected with *E. maxima* was initially higher than that of infected 15I birds, however at later time points, this was reversed and line 15I birds had higher serum IL-10 than infected C.B12 birds. Serum IL-10 in infected susceptible line 15I birds also continued to increase to a much greater extent than in line C.B12 (Figure 5-11A).

Following *E. tenella* infection, there were no differences in serum IL-10 between the two lines but compared to uninfected birds significant ($p<0.05$) increases in serum IL-10 were observed (Figure 5-11B).

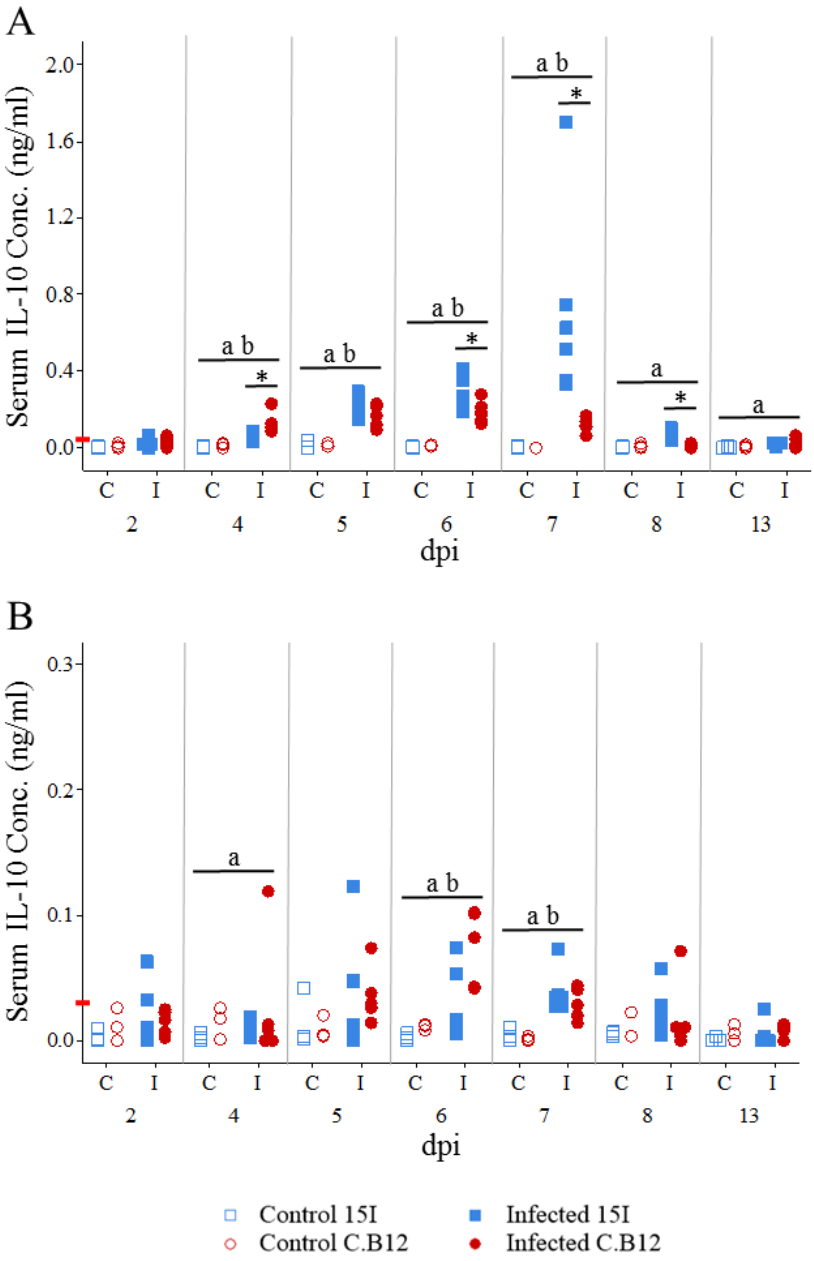

Figure 5-11: Serum IL-10 in line 15I and C.B12 chickens infected with *E. maxima* (A) or *E. tenella* (B). Three-week-old birds were orally inoculated with 100 *E. maxima* oocysts, 200 *E. tenella* oocysts or sterile water (control birds) and serum collected at culling at various time points post infection. Data are presented as individual birds. Red tick mark on the *y* axis indicates the detection threshold of the assay (30 pg/ml). Significant differences between the two lines given the same treatment are indicated by an asterisk, between 15I control and infected birds by an 'a' and between C.B12 control and infected birds by a 'b' ($p<0.05$, Mann-Whitney U Test); n=3 for control groups and n=5 or more for infected groups.

Serum IFNγ was measured by ELISA by Dr Sungwon Kim (Roslin Institute, UK). Following *E. maxima* infection, increased serum IFNγ was observed in both lines. Early after infection, the resistant line C.B12 birds had higher serum IFNγ than line 15I birds although this was not significant. At later time points, infected line 15I birds displayed higher serum IFNγ than C.B12 birds (Figure 5-12A).

Serum IFNγ increased in both lines after *E. tenella* infection, but greater and earlier in susceptible line C.B12 birds (Figure 5-12B).

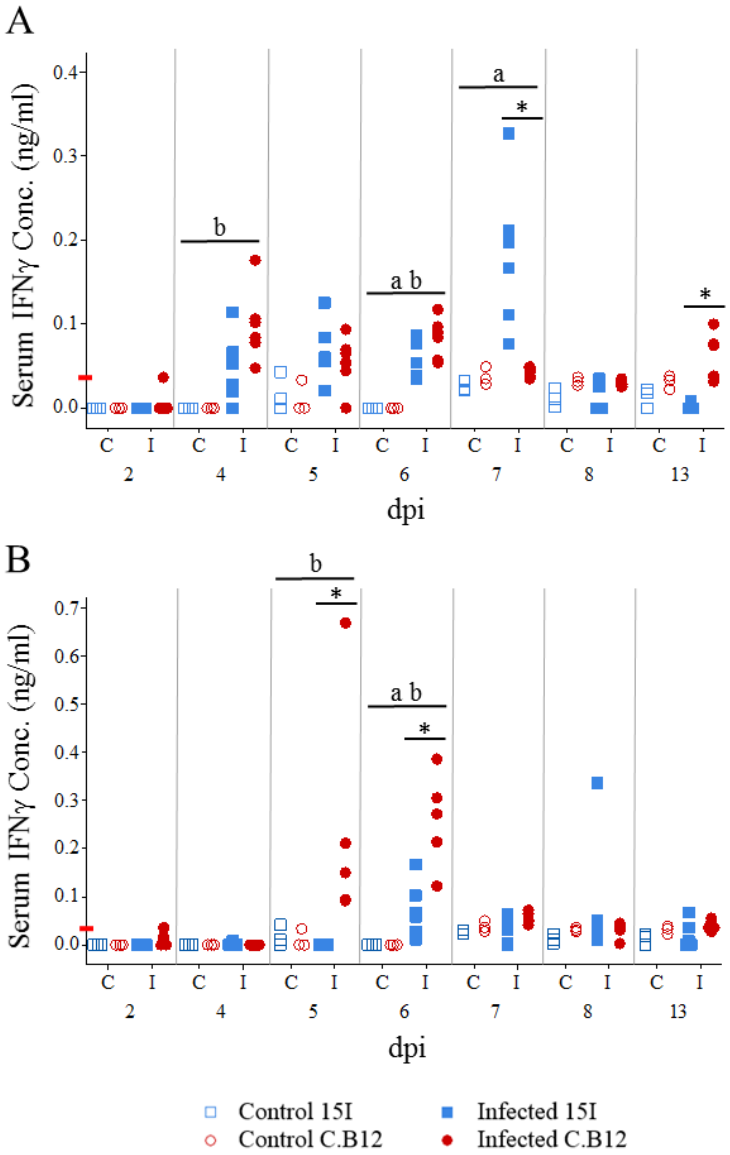

Figure 5-12: Serum IFNγ in line 15I and C.B12 chickens infected with *E. maxima* (A) or *E. tenella* (B). Three-week-old birds were orally inoculated with 100 *E. maxima* oocysts, 200 *E. tenella* oocysts or sterile water (control birds) and serum collected at culling at various time points post infection. Data are presented as individual birds. Red tick mark on the *y* axis indicates the detection threshold of the assay (31.25 pg/ml). Significant differences between the two lines given the same treatment are indicated by an asterisk, between 15I control and infected birds by an 'a' and between C.B12 control and infected birds by a 'b' ($p<0.05$, Mann-Whitney U Test); n=3 for control groups and n=5 or more for infected groups.

To examine the relationship between IL-10 and IFNγ in the serum and *Eimeria* parasite burden in the gut, the correlation between the two was examined (Table 5-5). Serum IL-10 and IFNγ correlated positively with *E. maxima* and *E. tenella* genome copy numbers in the jejunum and caecum of both chicken lines, although not always significantly.

Table 5-5*:* Spearman's rank correlation coefficients between serum IL-10 or IFNγ and *Eimeria* genome copy number in the jejunum following *E. maxima* infection (n=41 for line 15I and n=39 for line C.B12) and caecum following *E. tenella* infection (n=31 for line 15I and n=29 for line C.B12). Shown are the Spearman's correlation coefficient (ρ) for data of all infected birds pooled within lines from all time points. P values were calculated using the two-tailed t test and were considered significant at $p<0.05$ and highly significant at $p<0.001$.

	E. maxima		*E. tenella*	
	15I	C.B12	15I	C.B12
IL-10	0.74 ($p<0.001$)	0.61 ($p<0.001$)	0.33 ($p=0.07$)	0.55 ($p<0.05$)
IFNγ	0.52 ($p<0.001$)	0.19 ($p=0.25$)	0.55 ($p<0.05$)	0.34 ($p=0.07$)

As the patterns of serum IL-10 and IFNγ levels were similar during *E. maxima* infection, the Spearman's rank correlation coefficient was calculated between serum IL-10 and serum IFNγ. IL-10 and IFNγ were found to be positively correlated at $\rho=0.66$ ($p<0.001$) for *E. maxima* and $\rho=0.49$ ($p<0.001$) for *E. tenella* infection.

To examine how closely related the concentration of IL-10 in the serum and tissue were to one another and to the level of mRNA expressed in the gut, the correlations between them was calculated. IL-10 levels in gut tissue and serum, and *IL10* mRNA expression in the gut correlated positively with one another following both *E. maxima* (Table 5-6) and *E. tenella* infection (Table 5-7).

Table 5-6: Spearman's rank correlation coefficients between serum IL-10 and tissue IL-10 as quantified by ELISA and *IL10* mRNA expression in the jejunum following *E. maxima* infection. Shown are the Spearman's correlation coefficients (ρ) for data of control and infected birds from 2, 4, 6, 8 and 13 dpi, calculated between the mean serum or tissue IL-10 concentration of duplicate wells and mean corrected 40-Ct of triplicate wells in individual birds. P values were calculated using the two-tailed t test and were considered significant at $p<0.05$ and highly significant at $p<0.001$; n=85.

	Serum	Tissue	mRNA
Serum		0.61 ($p<0.001$)	0.51 ($p<0.001$)
Tissue	0.61 ($p<0.001$)		0.69 ($p<0.001$)
mRNA	0.51 ($p<0.001$)	0.69 ($p<0.001$)	

Table 5-7: Spearman's rank correlation coefficients between serum IL-10 and tissue IL-10 as quantified by ELISA and *IL10* mRNA expression in the caecum following *E. tenella* infection. Shown are the Spearman's correlation coefficients (ρ) for data of control and infected birds from 2, 4, 6, 8 and 13 dpi, calculated between the mean serum or tissue IL-10 concentration of duplicate wells and mean corrected 40-Ct of triplicate wells in individual birds. P values were calculated using the two-tailed t test and were considered significant at $p<0.05$ and highly significant at $p<0.001$; n=85.

	Serum	Tissue	mRNA
Serum		0.35 ($p<0.05$)	0.26 ($p<0.05$)
Tissue	0.35 ($p<0.05$)		0.64 ($p<0.001$)
mRNA	0.26 ($p<0.05$)	0.64 ($p<0.001$)	

To examine how closely the level of IFNγ in the serum reflected that of *IFNG* expression in the gut following infection, the Spearman's rank correlation coefficient was calculated between serum IFNγ and *IFNG* mRNA expression in the jejunum and caecum following *E. maxima* and *E. tenella* infection respectively. IFNγ in the serum correlated positively with *IFNG* mRNA expression in the gut at ρ=0.71 ($p<0.001$) for *E. maxima* and ρ=0.68 for *E. tenella* infection.

5.2.6 Changes to cell subpopulations in the gut of *E. maxima* and *E. tenella* infected 15I and C.B12 chickens.

To assess differences to the subpopulation of immune cells in the gut during *Eimeria* infection between the two chicken lines, ICC analysis was performed on snap frozen tissues and cell populations scored as in Section 2.6.2. The results are shown here alongside histology images of the gut. Sections were cut and stained from tissues collected at 2, 7 and 13 dpi.

Following *E. maxima* infection, no parasites were observed in the jejunum of infected birds of either line at 2 or 13 dpi, however *E. maxima* gamonts containing microgametes and macrogametes were observed at 7 dpi (shown in Figure 5-13 for line 15I). Gamonts were mainly located throughout the villi of the jejunum, either in the epithelium or sitting in the lamina propria just underneath the epithelial layer.

As with *E. maxima* infection, *E. tenella* was observed in the caecum of infected birds at 7 dpi only (shown in Figure 5-14 for line C.B12). High numbers of *E. tenella* gamonts were observed throughout the crypt epithelium of caeca from both lines.

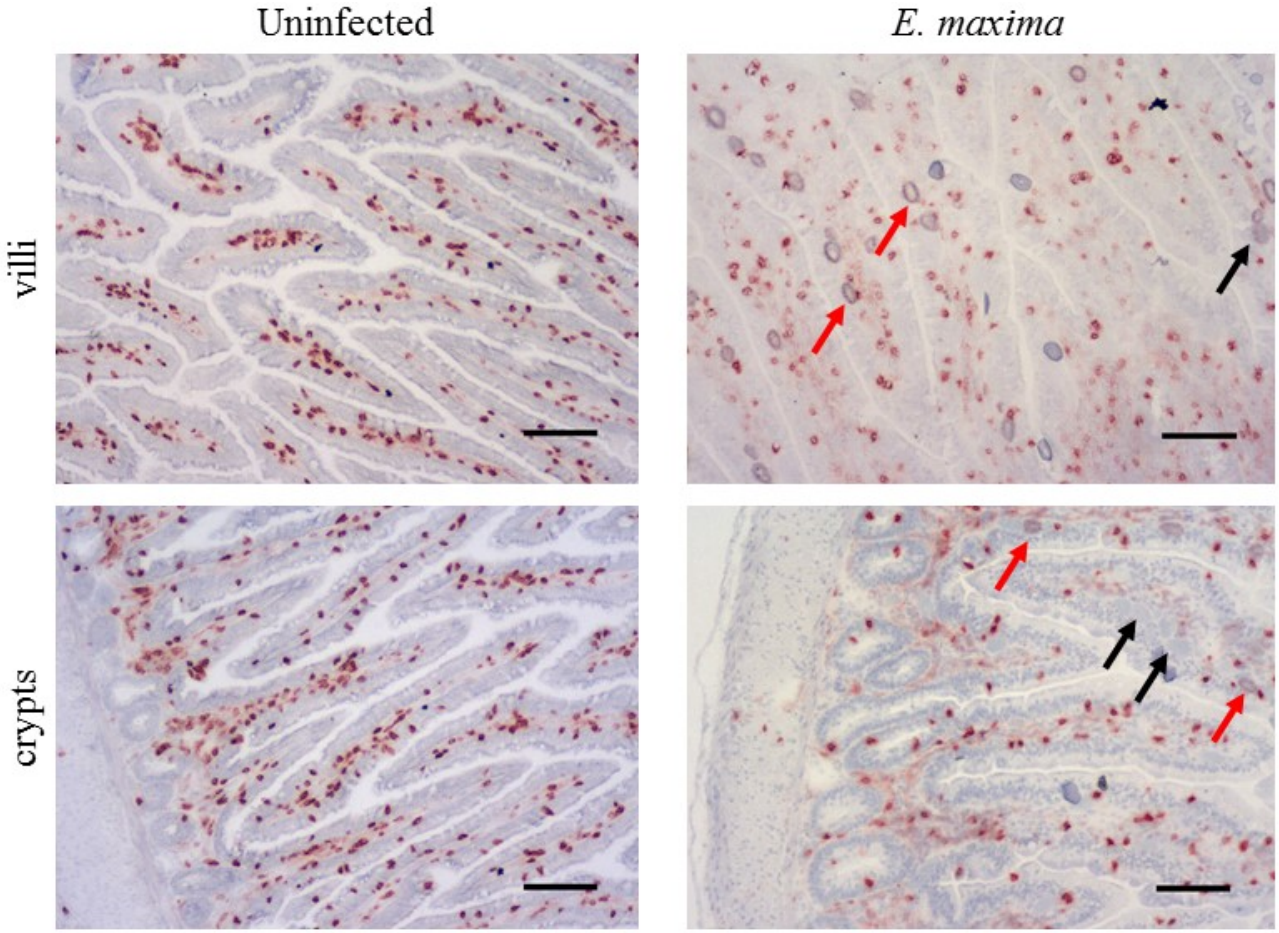

Figure 5-13: Control and *E. maxima*-infected jejunum villi and crypt regions from line 15I chickens at 7 dpi. Red arrows indicate *E. maxima* macrogametes. Black arrows indicate *E. maxima* microgametes. Red AEC stain indicates $CD25^{+}$ cells. Bars represent 100 µm.

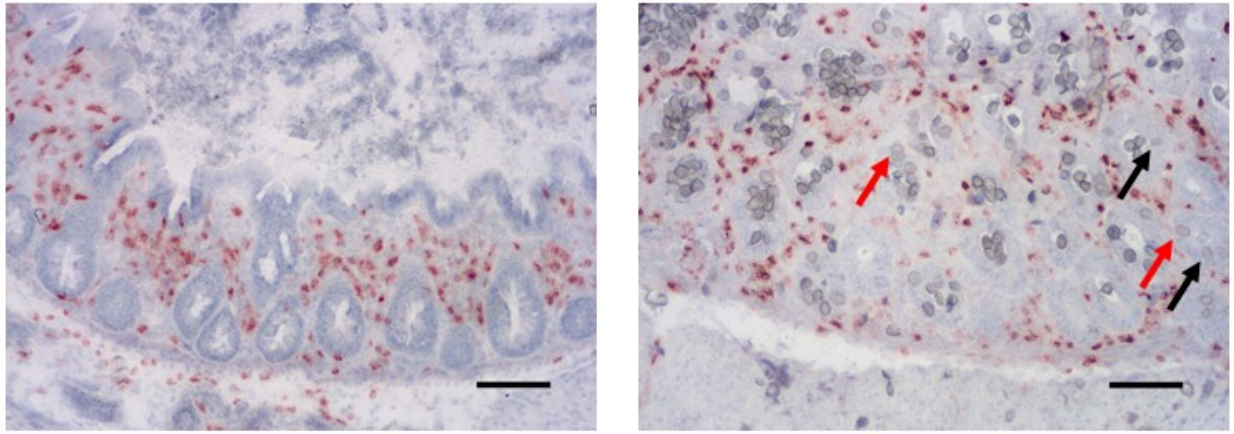

Figure 5-14: Control and *E. tenella*-infected caeca from line C.B12 chickens at 7 dpi. Red arrows indicate *E. maxima* macrogametes. Black arrows indicate *E. maxima* microgametes. Red AEC stain indicates $CD25^{+}$ cells. Bars represent 100 µm.

At 2 dpi with *E. maxima* and *E. tenella* infection, no apparent differences were observed in the cell subpopulations between infected and control birds or between the two lines therefore only sections cut from 7 and 13 dpi tissues were scored for each cell subpopulation. In the jejunum of control 15I and C.B12 birds, the majority of $CD4^+$ cells were LPLs and in general, with the exception of areas containing $CD4^+$ clusters, were evenly spaced throughout the lamina propria of both crypts and villi. In line 15I birds, $CD4^+$ IELs were few whereas line C.B12 seemed to have slightly higher numbers of $CD4^+$ IELs. In both lines, $CD4^+$ IELs and LPLs exhibited a rounded morphology. Clusters of $CD4^+$ LPLs were observed in some control birds of both lines. In infected susceptible line 15I birds, all birds had areas of crypt that were nearly completely stained with $CD4^+$ cells however this was not where the majority of the *E. maxima* gamonts were observed, at least at this stage of the parasite's life cycle (Figure 5-15, Figure 5-16 and Figure 5-17).

In control caeca of both lines, $CD4^+$ LPL clusters were observed in the majority of birds from both lines. Otherwise, $CD4^+$ LPLs were scattered evenly throughout the lamina propria and were rounded in morphology. Very few caecal $CD4^+$ IELs were observed in either of the lines, in control or *E. tenella*-infected birds. Following *E. tenella* infection huge increases of $CD4^+$ cells were observed in both lines (Figure 5-15 and Figure 5-18).

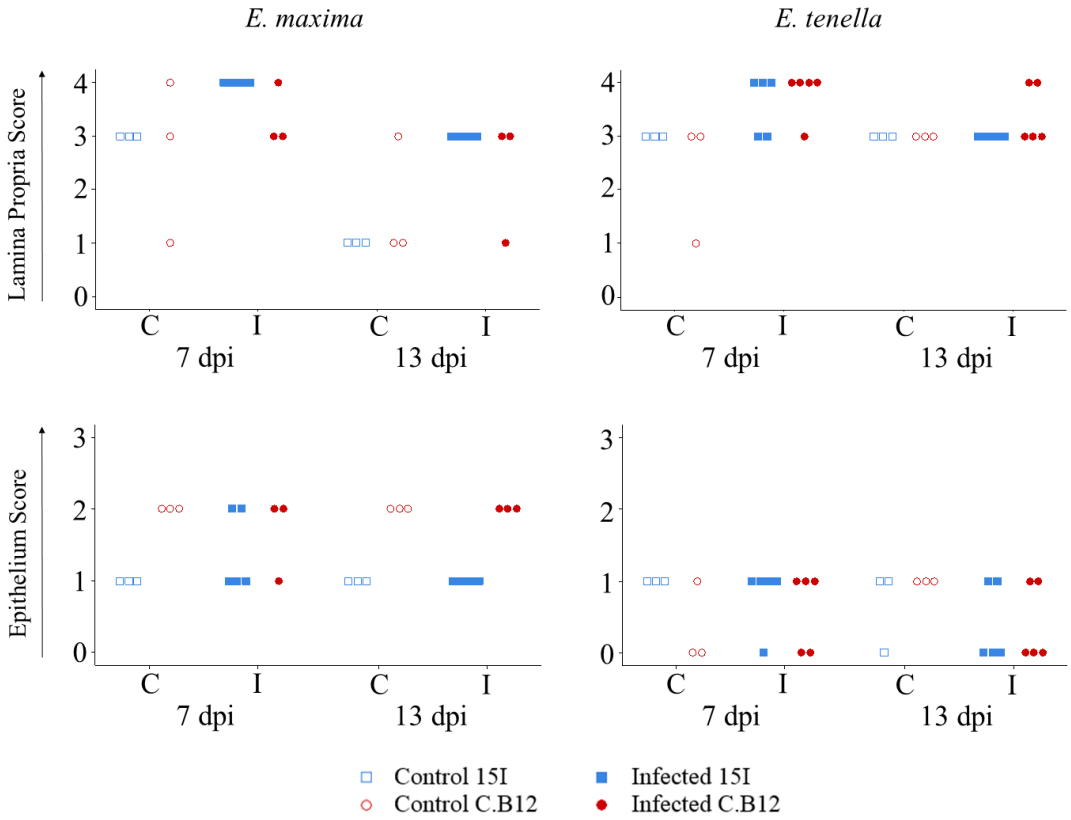

Figure 5-15: CD4$^+$ cells in the jejunum of *E. maxima*-infected and mid-caecum of *E. tenella*-infected lines 15I and C.B12 chickens. Three-week old chicks were orally inoculated with 100 *E. maxima* oocysts, 200 *E. tenella* oocysts or sterile water and samples collected for histology at 7 and 13 dpi. Cell populations were scored as in section 2.6.2. Each individual dot represents data from one bird. C; Control. I; Infected.

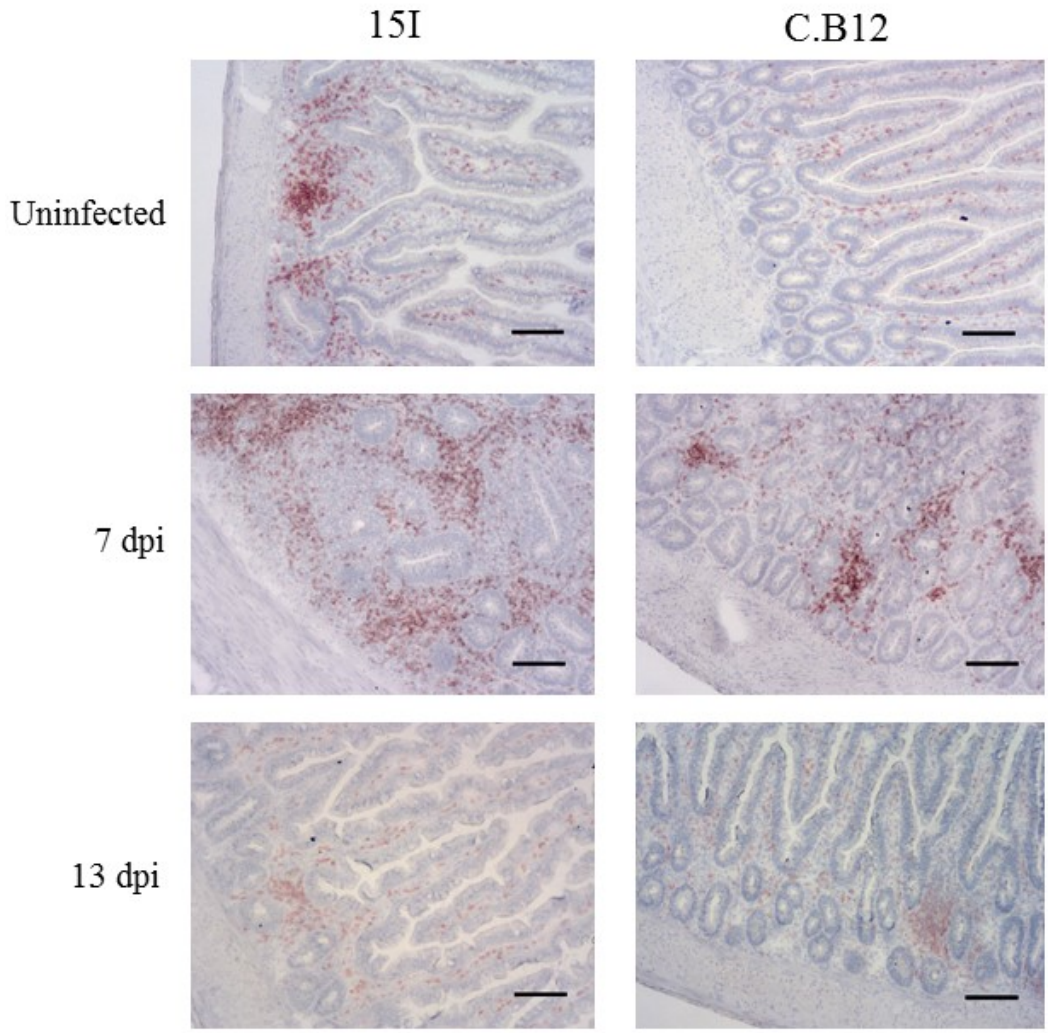

Figure 5-16: $CD4^+$ cells in the crypts of the jejunum of *E. maxima*-infected line 15I and C.B12 birds. Three-week-old birds were orally inoculated with 100 *E. maxima* oocysts and samples collected for histology at 7 and 13 dpi. Red AEC stain indicates $CD4^+$ cells. Bars represent 100 µm.

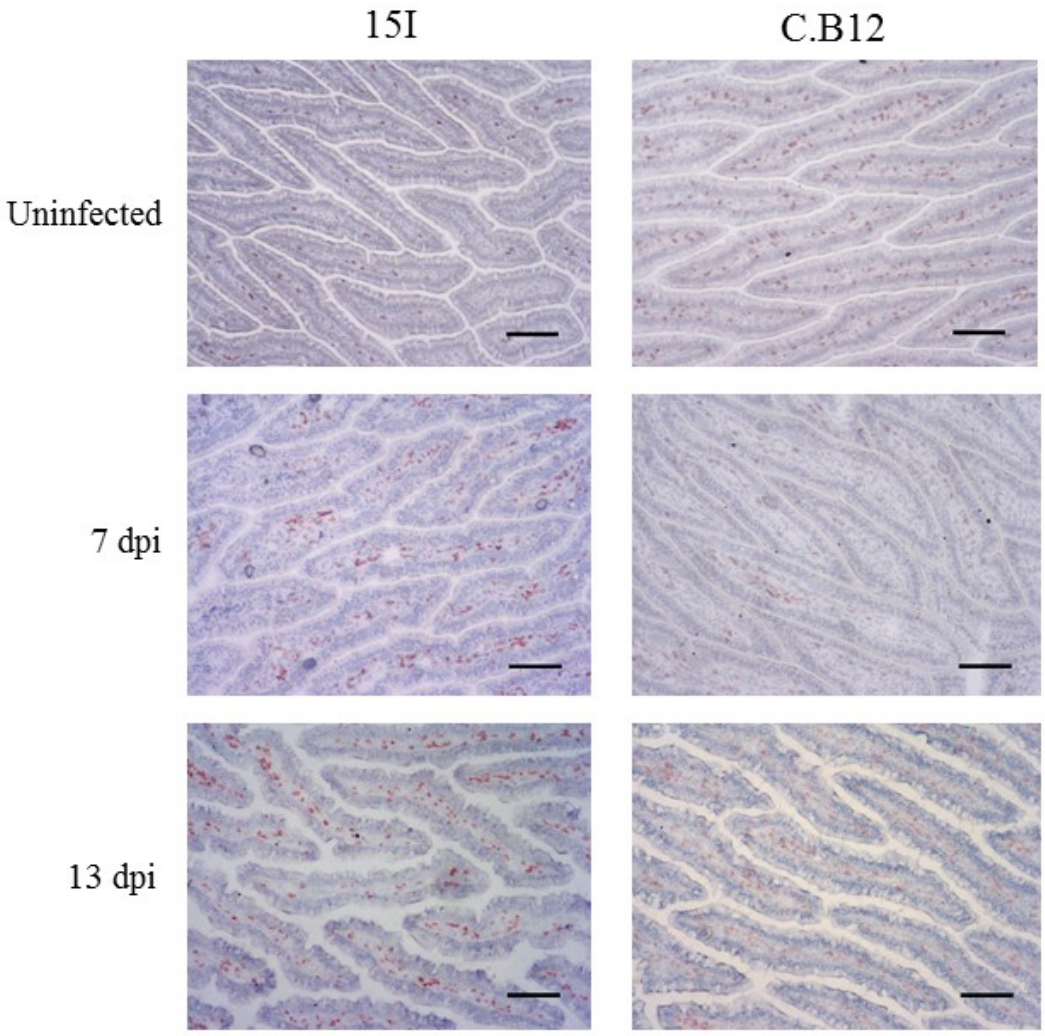

Figure 5-17: $CD4^+$ cells in the villi of the jejunum of *E. maxima*-infected line 15I and C.B12 birds. Three-week-old birds were orally inoculated with 100 *E. maxima* oocysts and samples collected for histology at 7 and 13 dpi. Red AEC stain indicates $CD4^+$ cells. Bars represent 100 μm.

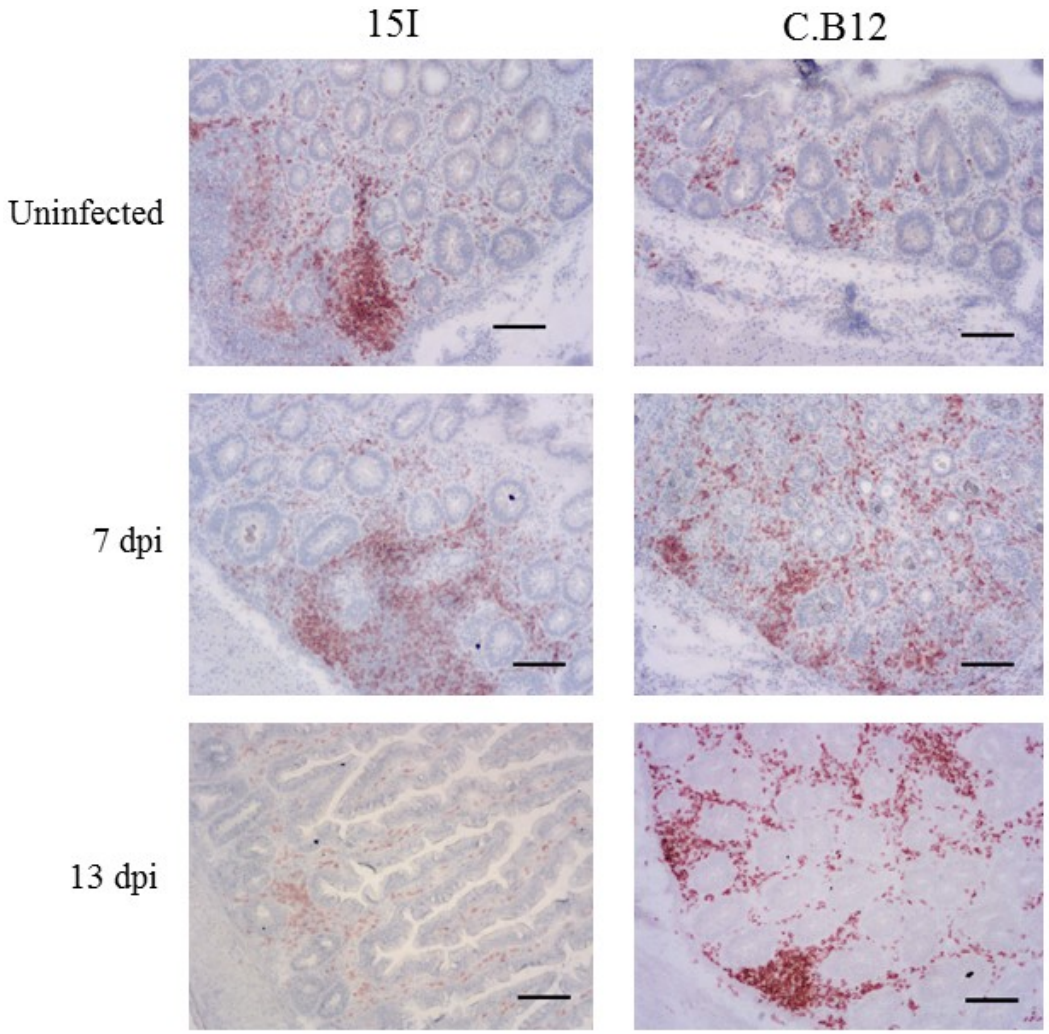

Figure 5-18: $CD4^+$ cells in the caecum of *E. tenella*-infected line 15I and C.B12 birds. Three-week-old birds were orally inoculated with 200 *E. tenella* oocysts and samples collected for histology at 7 and 13 dpi. Red AEC stain indicates $CD4^+$ cells. Bars represent 100 μm.

In control birds of both lines, CD8α$^+$ cells were LPLs and IELs scattered throughout the jejunum, with small clusters throughout the crypts and villi. Overall there was no difference in the CD8α$^+$ cell population between the two lines, in either control or infected birds. Following *E. maxima* infection, high numbers of infiltrating CD8α$^+$ LPLs were observed in both lines at 7 dpi, but by 13 dpi, only smaller clusters of CD8α$^+$ LPLs were observed (Figure 5-19, Figure 5-20 and Figure 5-21). At 7 dpi, it was observed that the anti-CD8α antibody used (clone 3-298) resulted in staining of *E. maxima* gamonts (Figure 5-21). This was not observed in sections stained with isotype control antibodies. To account for this, stained gamonts were excluded when scoring was performed on the sections.

In control birds of both lines, CD8α$^+$ LPLs and IELs were scattered throughout the caeca. CD8α$^+$ LPLs increased to a greater extent in susceptible line C.B12 compared with line 15I following *E. tenella* infection (Figure 5-19 and Figure 5-22).

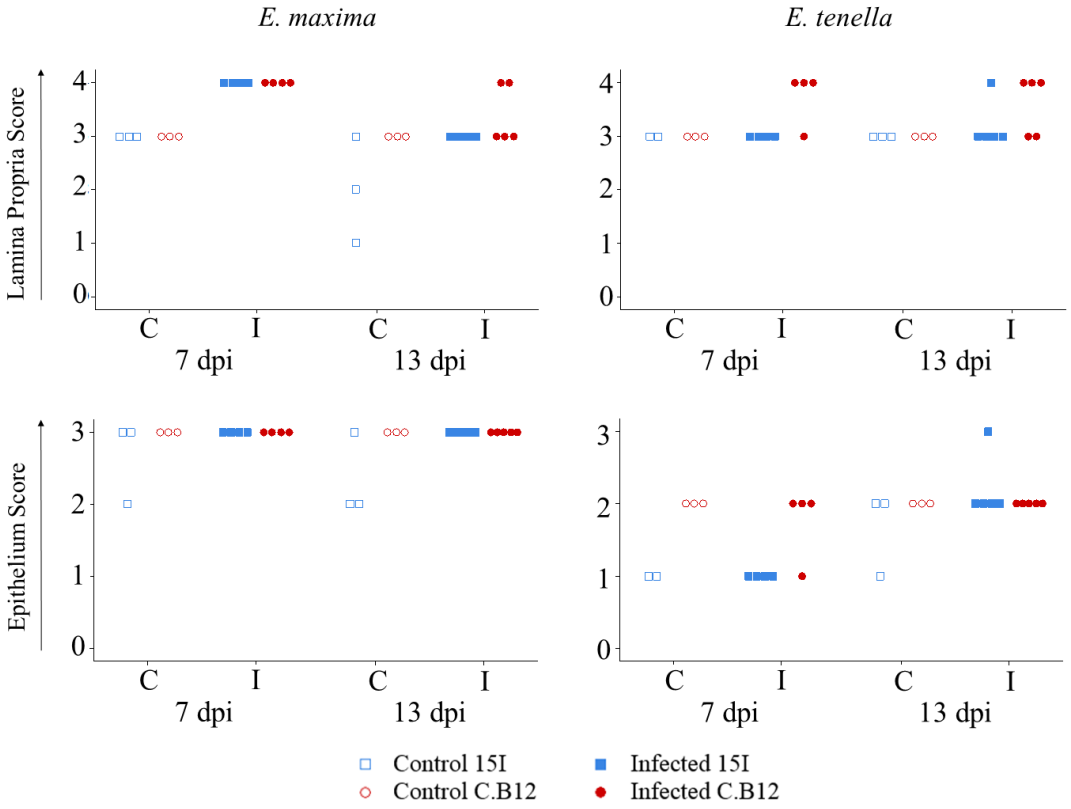

Figure 5-19: CD8α^{+} cells in the jejunum of *E. maxima*-infected and mid-caecum of *E. tenella*-infected lines 15I and C.B12 chickens. Three-week old chicks were orally inoculated with 100 *E. maxima* oocysts, 200 *E. tenella* oocysts or sterile water and samples collected for histology at 7 and 13 dpi. Cell populations were scored as in section 2.6.2. Each individual dot represents data from one bird. C; Control. I; Infected.

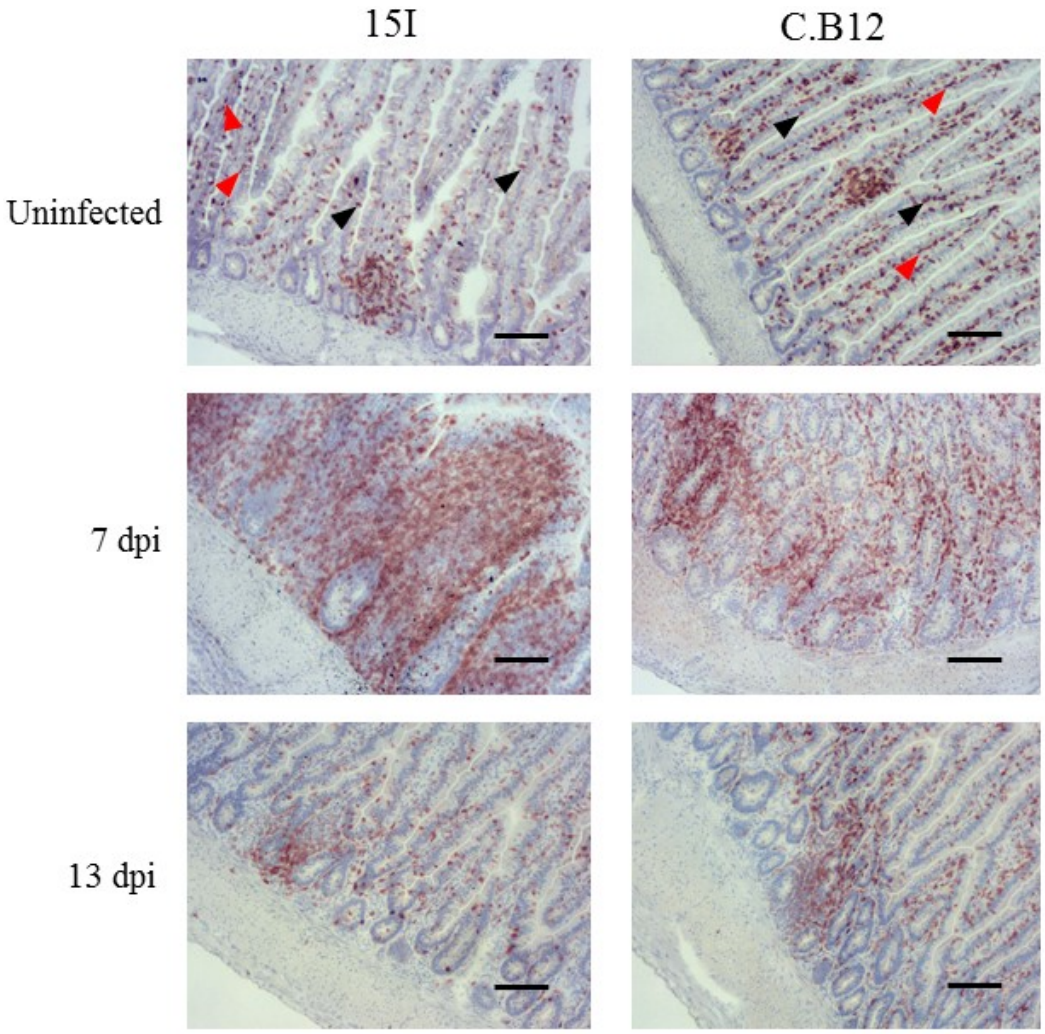

Figure 5-20: CD8α$^+$ cells in the crypts of the jejunum of *E. maxima*-infected line 15I and C.B12 birds. Three-week-old birds were orally inoculated with 100 *E. maxima* oocysts and samples collected for histology at 7 and 13 dpi. Red AEC stain indicates CD8α$^+$ cells. Black arrowheads indicate rounded CD8α$^+$ IELs. Red arrowheads indicate those which were elongated and pressed against the basal membrane. Bars represent 100 µm.

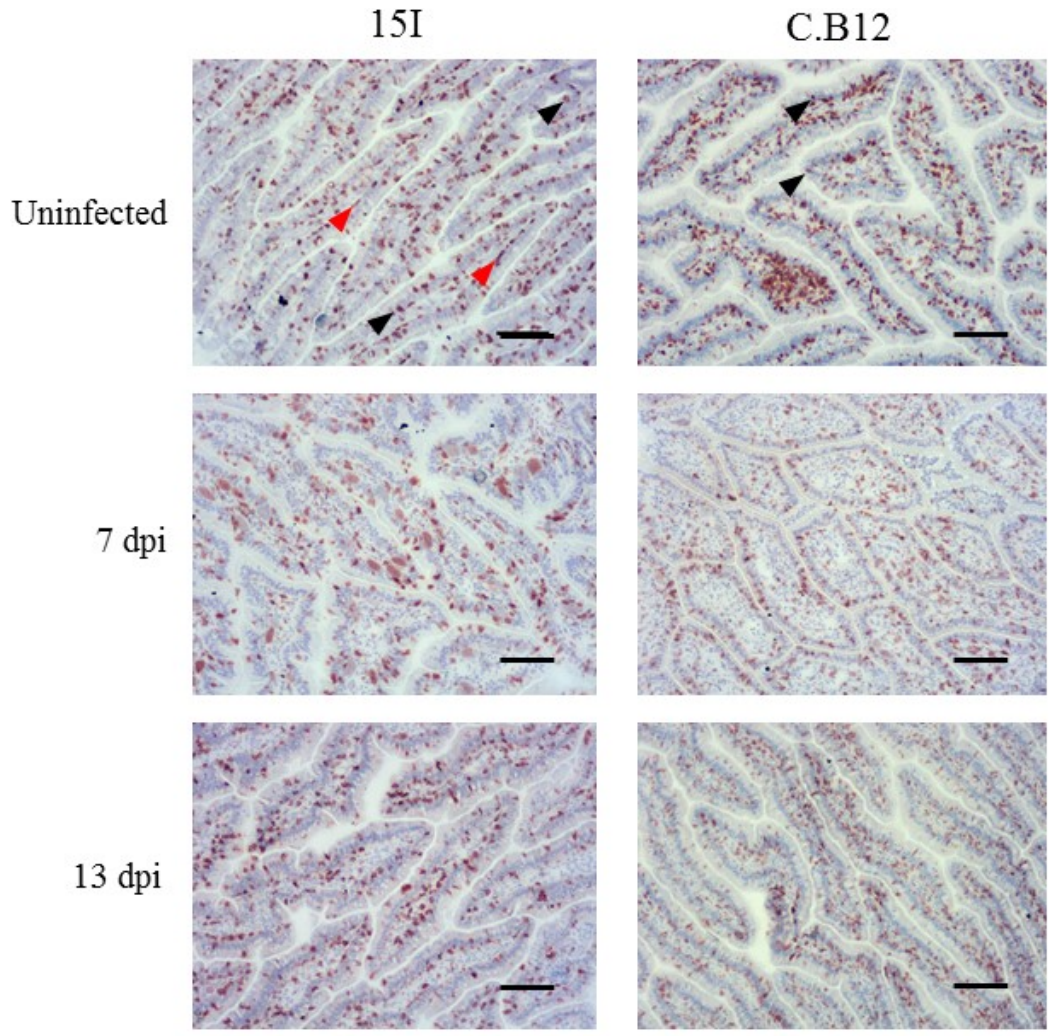

Figure 5-21: CD8α$^+$ cells in the villi of the jejunum of *E. maxima*-infected line 15I and C.B12 birds. Three-week-old birds were orally inoculated with 100 *E. maxima* oocysts and samples collected for histology at 7 and 13 dpi. Red AEC stain indicates CD8α$^+$ cells. Black arrowheads indicate rounded CD8α$^+$ IELs. Red arrowheads indicate those which were elongated and pressed against the basal membrane. Bars represent 100 µm.

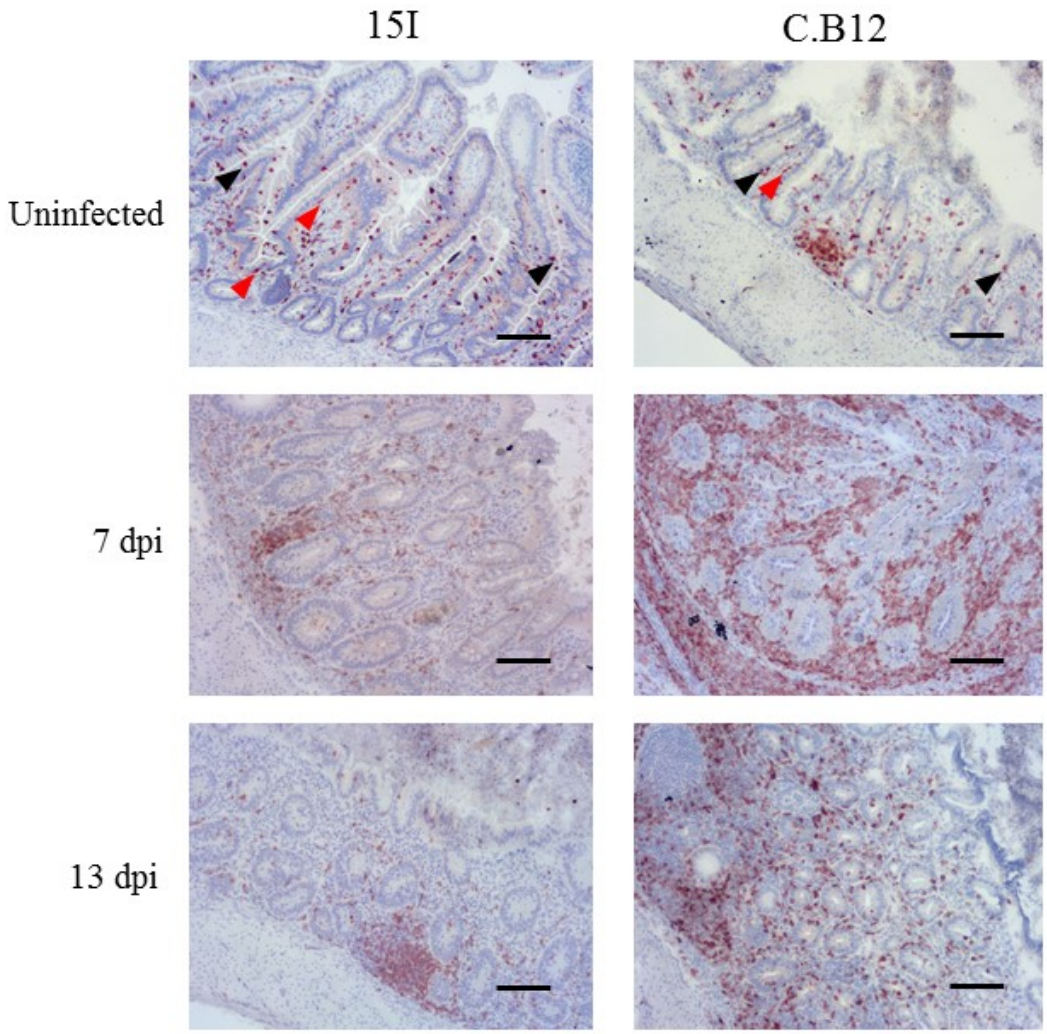

Figure 5-22*:* CD8α$^+$ cells in the caecum of *E. tenella*-infected line 15I and C.B12 birds. Three-week-old birds were orally inoculated with 200 *E. tenella* oocysts and samples collected for histology at 7 and 13 dpi. Red AEC stain indicates CD8α$^+$ cells. Black arrowheads indicate rounded CD8α$^+$ IELs. Red arrowheads indicate those which were elongated and pressed against the basal membrane. Bars represent 100 µm.

In control jejunum from both lines, the majority of TCRγδ$^+$ cells were IELs. Very few TCRγδ$^+$ cells were LPLs but those that were present were mainly located just underneath the epithelium of both crypts and villi and were smaller in size than TCRγδ$^+$ IELs although the majority of TCRγδ$^+$ cells observed were rounded in morphology. Following *E. maxima* infection, no major changes were observed to the population of TCRγδ$^+$ cells in either line and no differences between the lines in the numbers of TCRγδ$^+$ cells were observed (Figure 5-23, Figure 5-24 and Figure 5-25).

In control caeca of both lines, the majority of TCRγδ$^+$ cells were IELs but a few TCRγδ$^+$ LPLs were scattered throughout the lamina propria. Following *E. tenella* infection, elevated numbers of TCRγδ$^+$ LPLs and IELs were observed in some infected and control birds of both lines (Figure 5-23 and Figure 5-26).

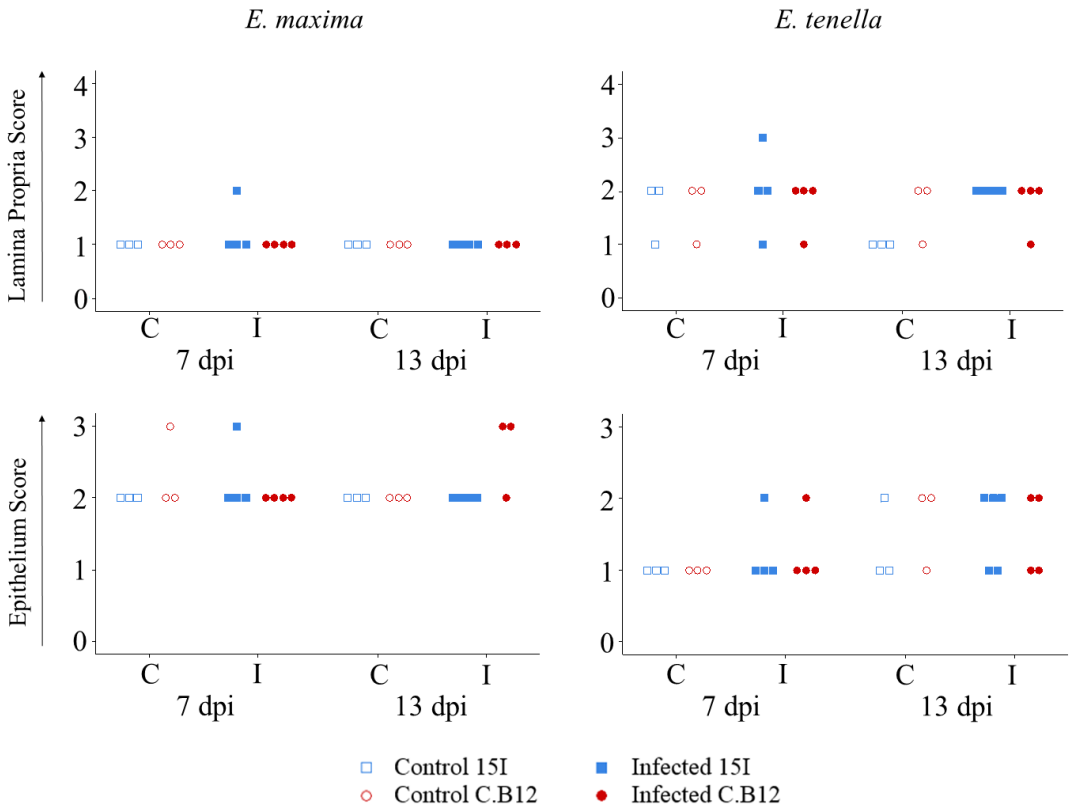

Figure 5-23: TCRγδ^{+} cells in the jejunum of *E. maxima*-infected and mid-caecum of *E. tenella*-infected lines 15I and C.B12 chickens. Three-week old chicks were orally inoculated with 100 *E. maxima* oocysts, 200 *E. tenella* oocysts or sterile water and samples collected for histology at 7 and 13 dpi. Cell populations were scored as in section 2.6.2. Each individual dot represents data from one bird. C; Control. I; Infected.

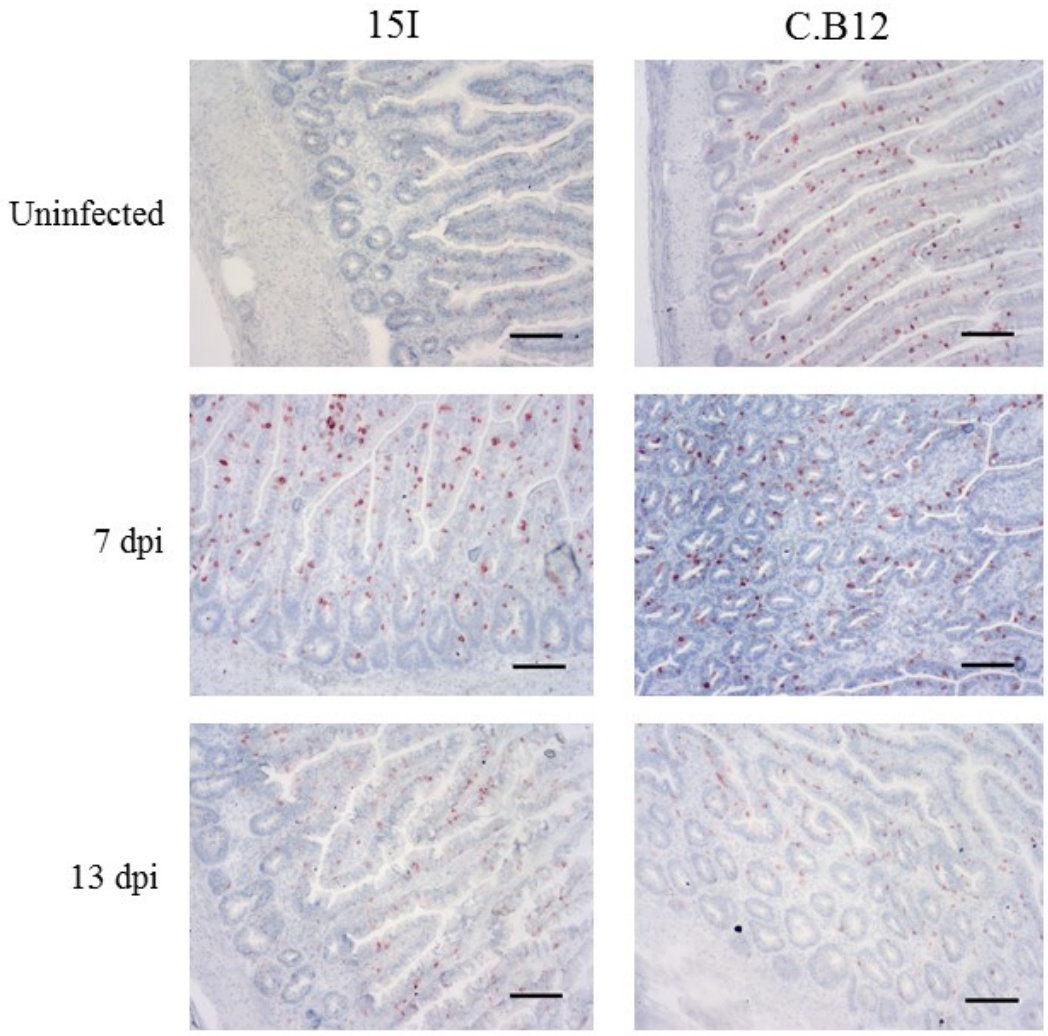

Figure 5-24: TCR$\gamma\delta^+$ cells in the crypts of the jejunum of *E. maxima*-infected line 15I and C.B12 birds. Three-week-old birds were orally inoculated with 100 *E. maxima* oocysts and samples collected for histology at 7 and 13 dpi. Red AEC stain indicates TCR$\gamma\delta^+$ cells. Bars represent 100 µm.

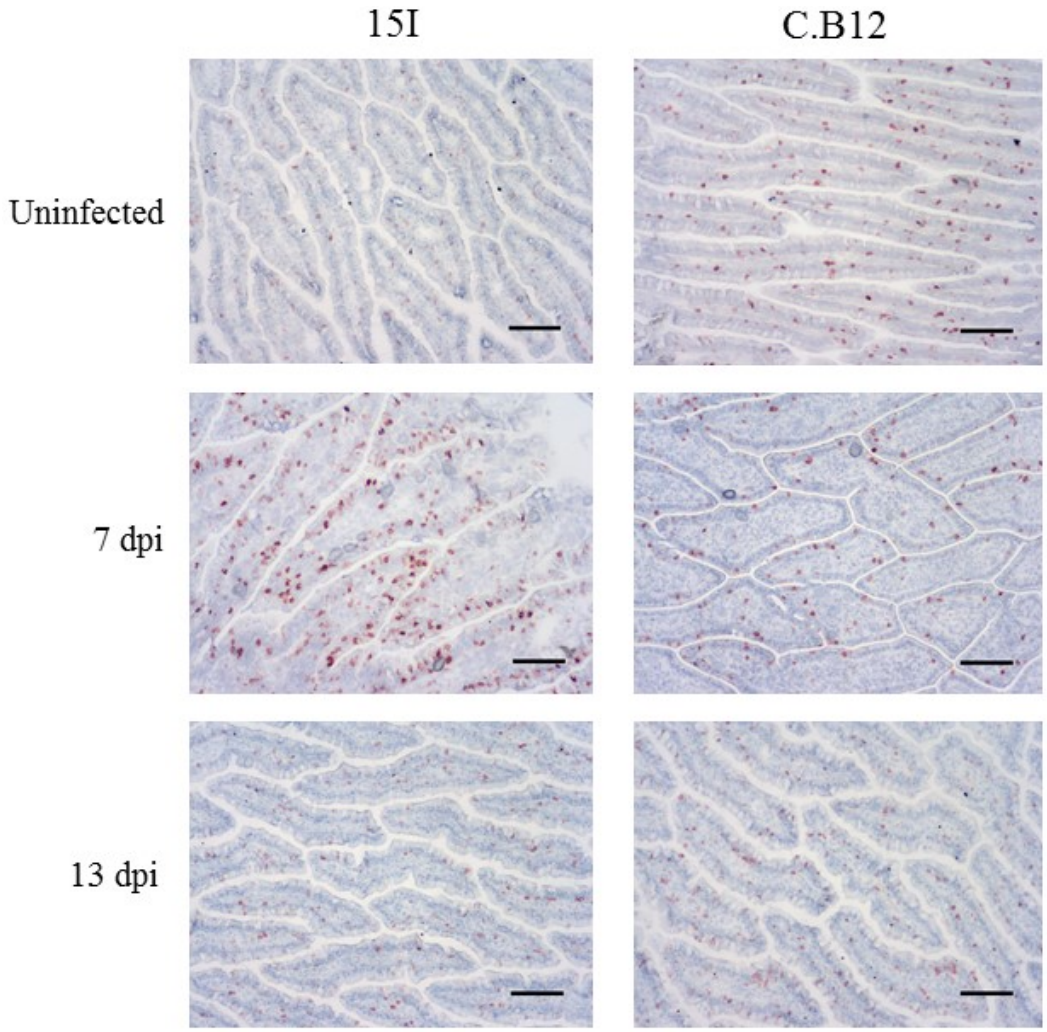

Figure 5-25: TCR$\gamma\delta^{+}$ cells in the villi of the jejunum of *E. maxima*-infected line 15I and C.B12 birds. Three-week-old birds were orally inoculated with 100 *E. maxima* oocysts and samples collected for histology at 7 and 13 dpi. Red AEC stain indicates TCR$\gamma\delta^{+}$ cells. Bars represent 100 μm.

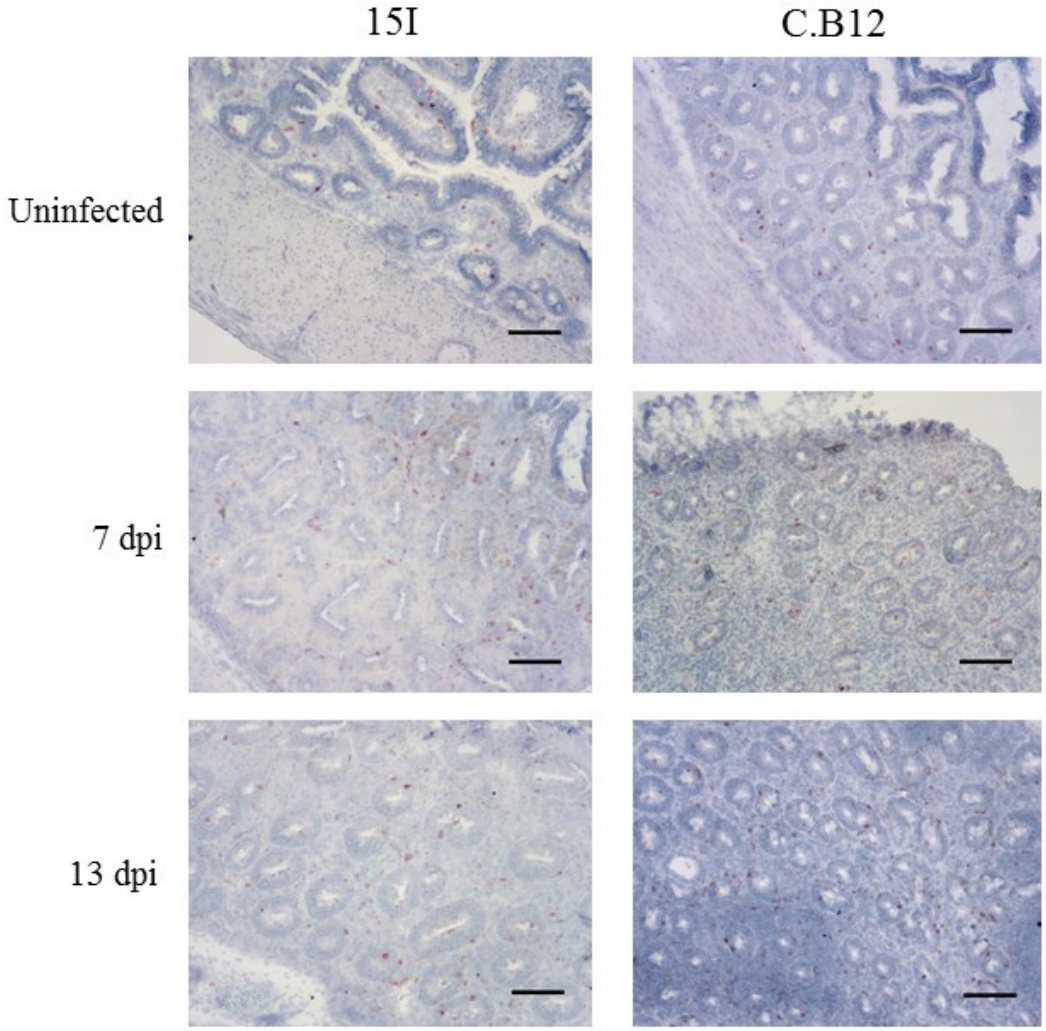

Figure 5-26: TCRγδ^{+} cells in the caecum of *E. tenella*-infected line 15I and C.B12 birds. Three-week-old birds were orally inoculated with 200 *E. tenella* oocysts and samples collected for histology at 7 and 13 dpi. Red AEC stain indicates TCRγδ^{+} cells. Bars represent 100 µm.

In the jejunum of control birds, the majority of TCRαβ$_1^+$ cells were LPLs and many clusters of TCRαβ$_1^+$ LPLs were observed as were areas of nearly complete staining (a score of 4) in both lines. TCRαβ$_1^+$ IELs were mainly scattered throughout the jejunum villus epithelium of both lines but were also present in the crypt epithelium. TCRαβ$_1^+$ LPLs are likely T cells that co-express CD4 or CD8. High numbers of TCRαβ$_1^+$ LPLs were observed in control and infected birds of both lines. Overall, there were no major changes in the numbers of TCRαβ$_1^+$ IELs as a result of *E. maxima* infection or between the two lines (Figure 5-27, Figure 5-28 and Figure 5-29).

As in the jejunum, caecal TCRαβ$_1^+$ cells were generally LPLs and likely to be T cells co-expressing CD4 or CD8. In many of the control birds, clusters of TCRαβ$_1^+$ LPLs were present but otherwise were scattered evenly throughout the lamina propria. Low numbers of TCRαβ$_1^+$ IELs were observed in the caecum of control birds. Following *E. tenella* infection, increased numbers of TCRαβ$_1^+$ LPLs and IELs were observed in the lamina propria of both lines compared to control birds (Figure 5-27 and Figure 5-30).

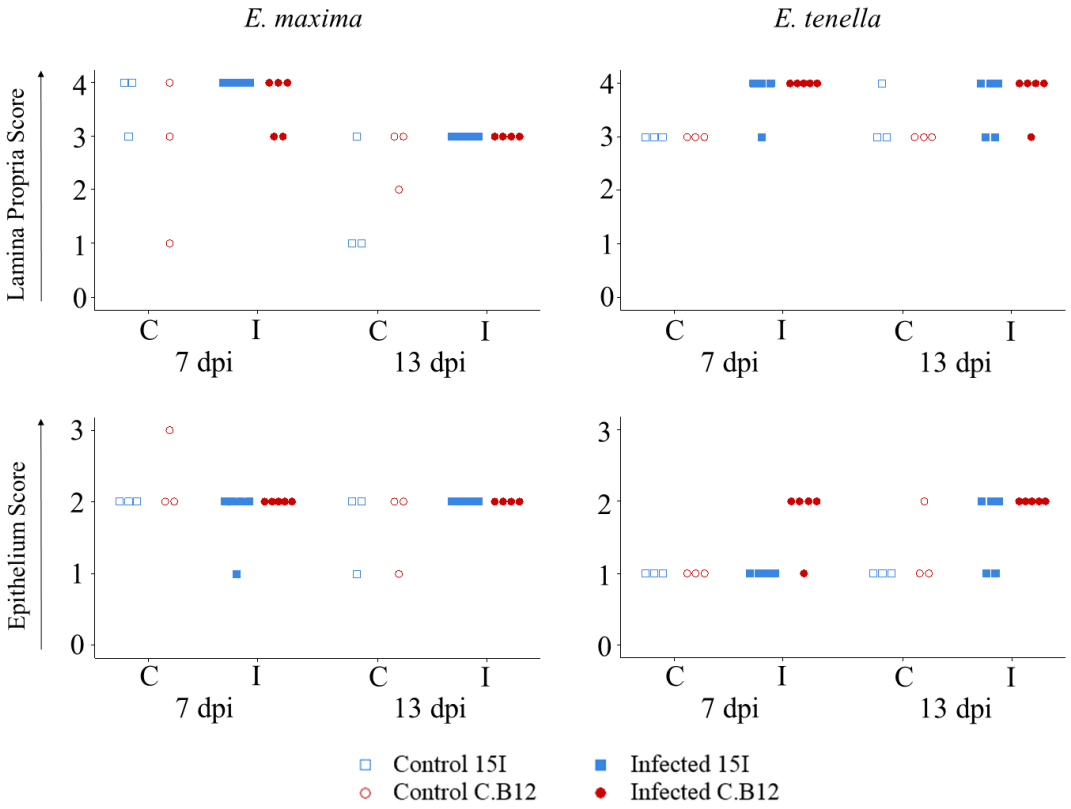

Figure 5-27: TCRαβ$_1^+$ cells in the jejunum of *E. maxima*-infected and mid-caecum of *E. tenella*-infected lines 15I and C.B12 chickens. Three-week old chicks were orally inoculated with 100 *E. maxima* oocysts, 200 *E. tenella* oocysts or sterile water and samples collected for histology at 7 and 13 dpi. Cell populations were scored as in section 2.6.2. Each individual dot represents data from one bird. C; Control. I; Infected.

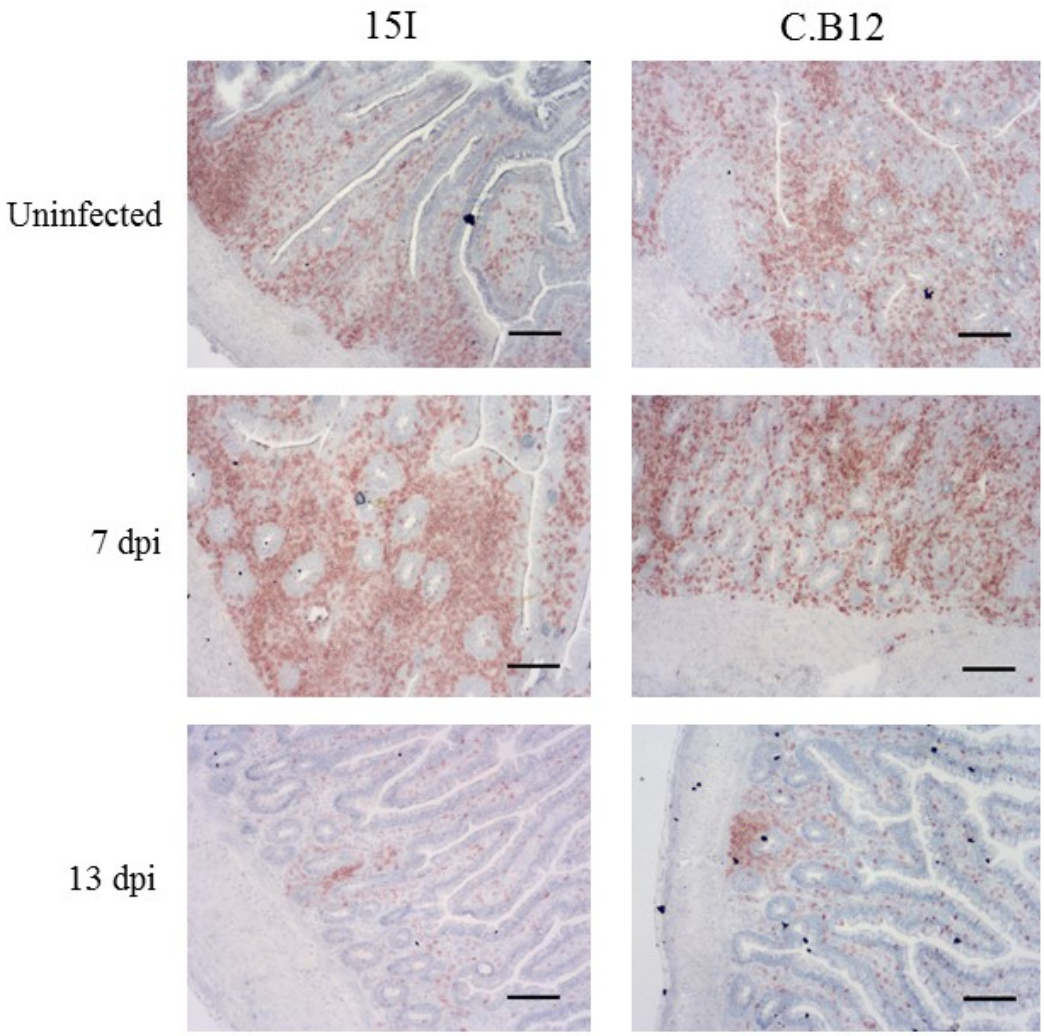

Figure 5-28: TCR$\alpha\beta_1^+$ cells in the crypts of the jejunum of *E. maxima*-infected line 15I and C.B12 birds. Three-week-old birds were orally inoculated with 100 *E. maxima* oocysts and samples collected for histology at 7 and 13 dpi. Red AEC stain indicates TCR$\alpha\beta_1^+$ cells. Bars represent 100 µm.

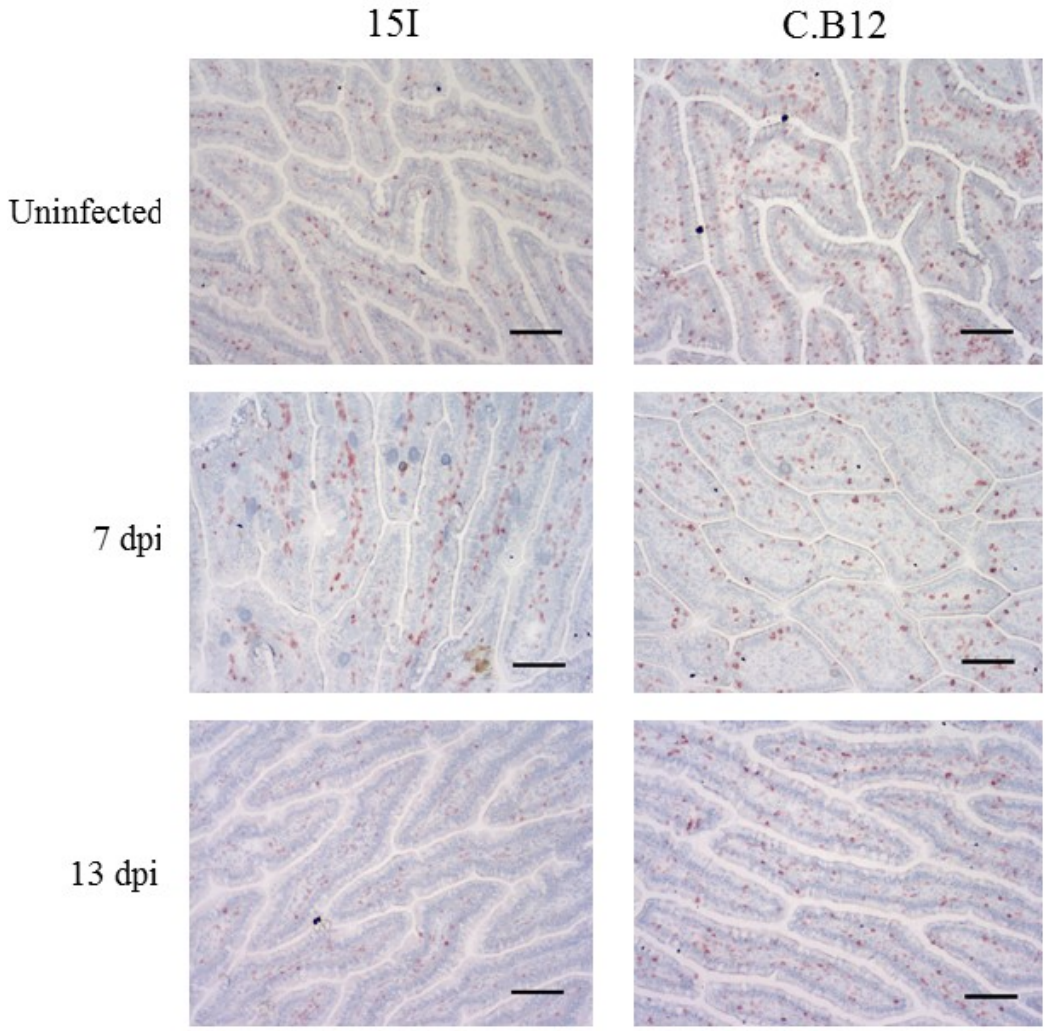

Figure 5-29: TCR$\alpha\beta_1^+$ cells in the villi of the jejunum of *E. maxima*-infected line 15I and C.B12 birds. Three-week-old birds were orally inoculated with 100 *E. maxima* oocysts and samples collected for histology at 7 and 13 dpi. Red AEC stain indicates TCR$\alpha\beta_1^+$ cells. Bars represent 100 µm.

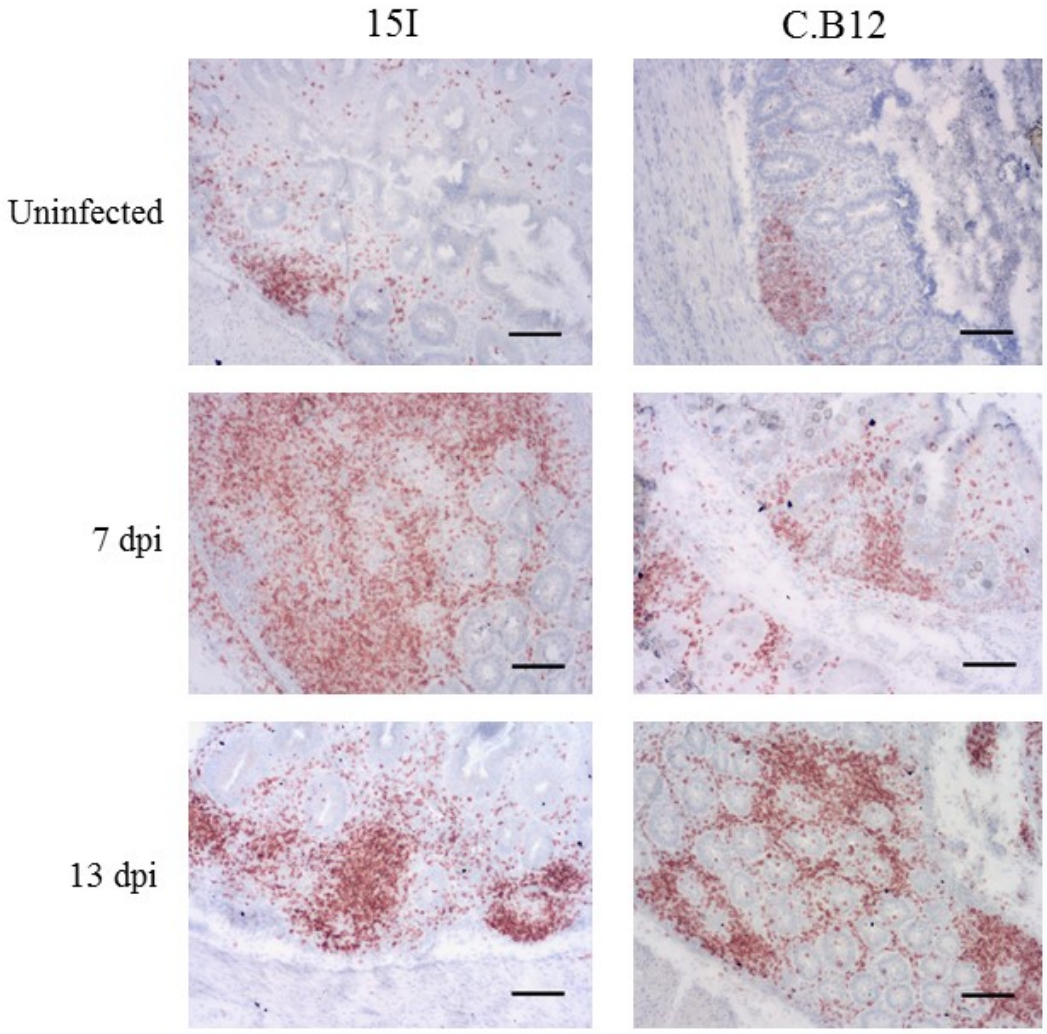

Figure 5-30: TCRαβ$_1^+$ cells in the caecum of *E. tenella*-infected line 15I and C.B12 birds. Three-week-old birds were orally inoculated with 200 *E. tenella* oocysts and samples collected for histology at 7 and 13 dpi. Red AEC stain indicates TCRαβ $_1^+$ cells. Bars represent 100 µm.

In the jejunum of control birds, $CD25^+$ cells were mainly scattered throughout the lamina propria in both the crypts and villi and it was not unusal to find clusters of $CD25^+$ LPLs in the crypts. $CD25^+$ IELs were also scattered throughout the epithelium of both the crypts and villi and were generally larger than $CD25^+$ LPLs. Both $CD25^+$ IELs and LPLs were rounded in shape; $CD25^+$ IELs likely represent epithelial NK cells (Göbel *et al*., 2001). Following *E. maxima* infection, large infiltrates of $CD25^+$ LPLs were observed in some but not all birds of both lines. Overall no major changes were observed in the $CD25^+$ IEL population in either line following infection (Figure 5-31, Figure 5-32 and Figure 5-33).

In control caeca of both lines, $CD25^+$ cells were mainly LPLs, although some $CD25^+$ IELs were scattered throughout the crypts and villi. As in the jejunum, $CD25^+$ cells in the caecum were rounded in morphology. $CD25^+$ LPLs increased in some but not all birds of both lines following *E. tenella* infection and overall, no differences were observed in the $CD25^+$ IELs population in either of the lines or in response to *E. tenella* infection (Figure 5-31 and Figure 5-34).

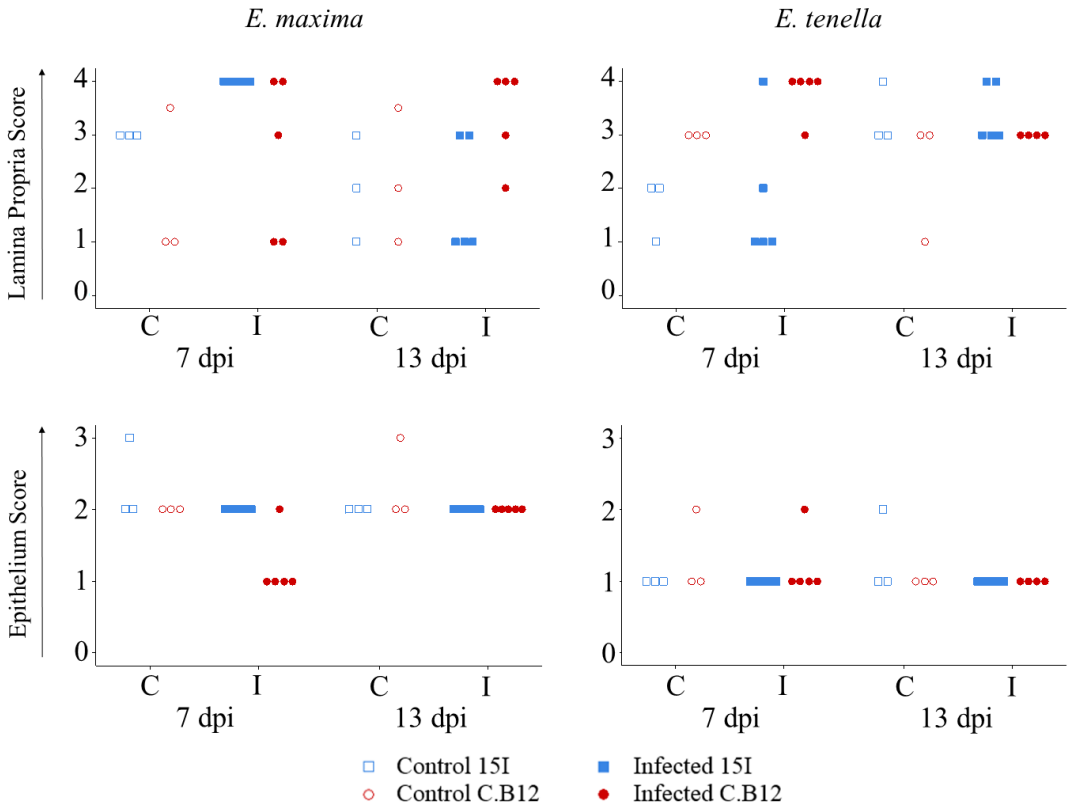

Figure 5-31: CD25^{+} cells in the jejunum of *E. maxima*-infected and mid-caecum of *E. tenella*-infected lines 15I and C.B12 chickens. Three-week old chicks were orally inoculated with 100 *E. maxima* oocysts, 200 *E. tenella* oocysts or sterile water and samples collected for histology at 7 and 13 dpi. Cell populations were scored as in section 2.6.2. Each individual dot represents data from one bird. C; Control. I; Infected.

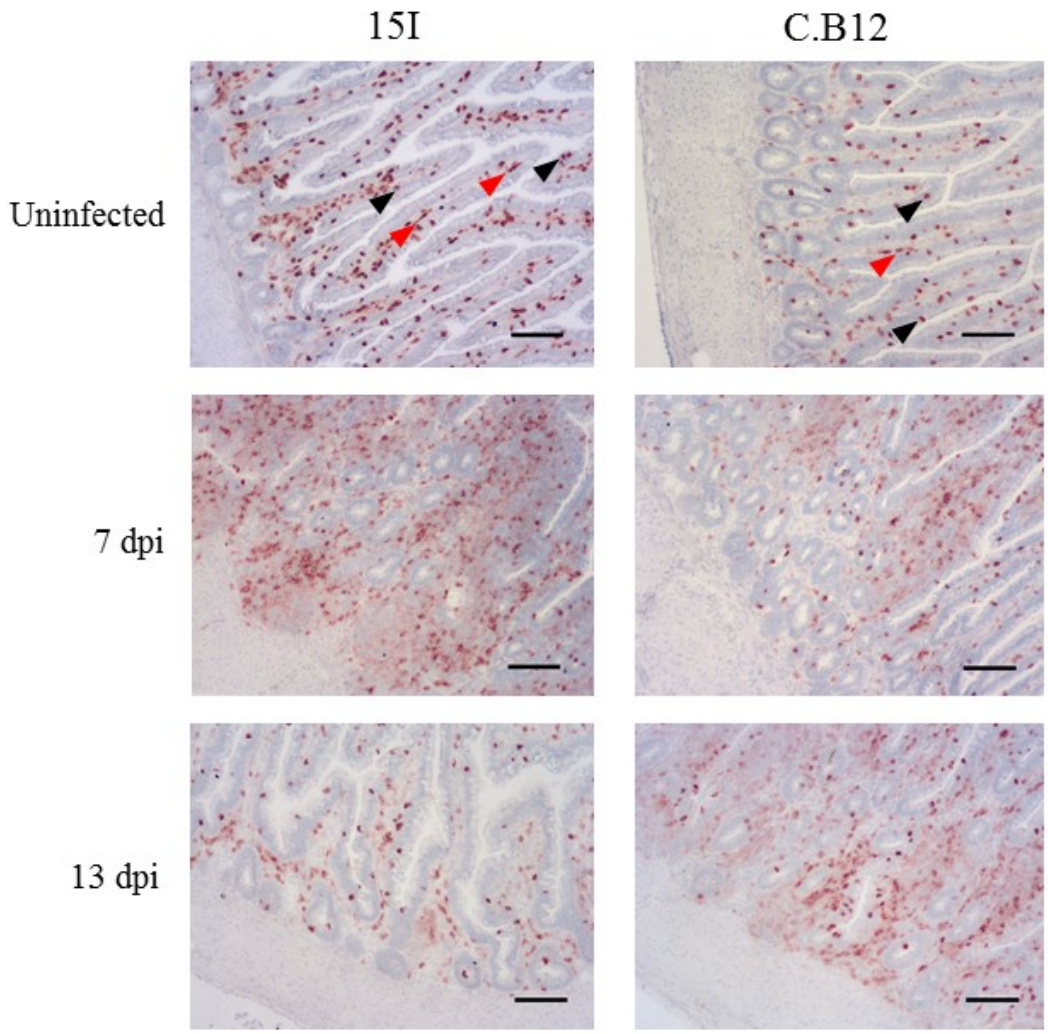

Figure 5-32: CD25$^+$ cells in the crypts of the jejunum of *E. maxima*-infected line 15I and C.B12 birds. Three-week-old birds were orally inoculated with 100 *E. maxima* oocysts and samples collected for histology at 7 and 13 dpi. Red AEC stain indicates CD25$^+$ cells. Black arrowheads indicate CD25$^+$ IELs of rounded morphology. Red arrowheads indicate CD25$^+$ cells pressed against the basal membrane. Bars represent 100 μm.

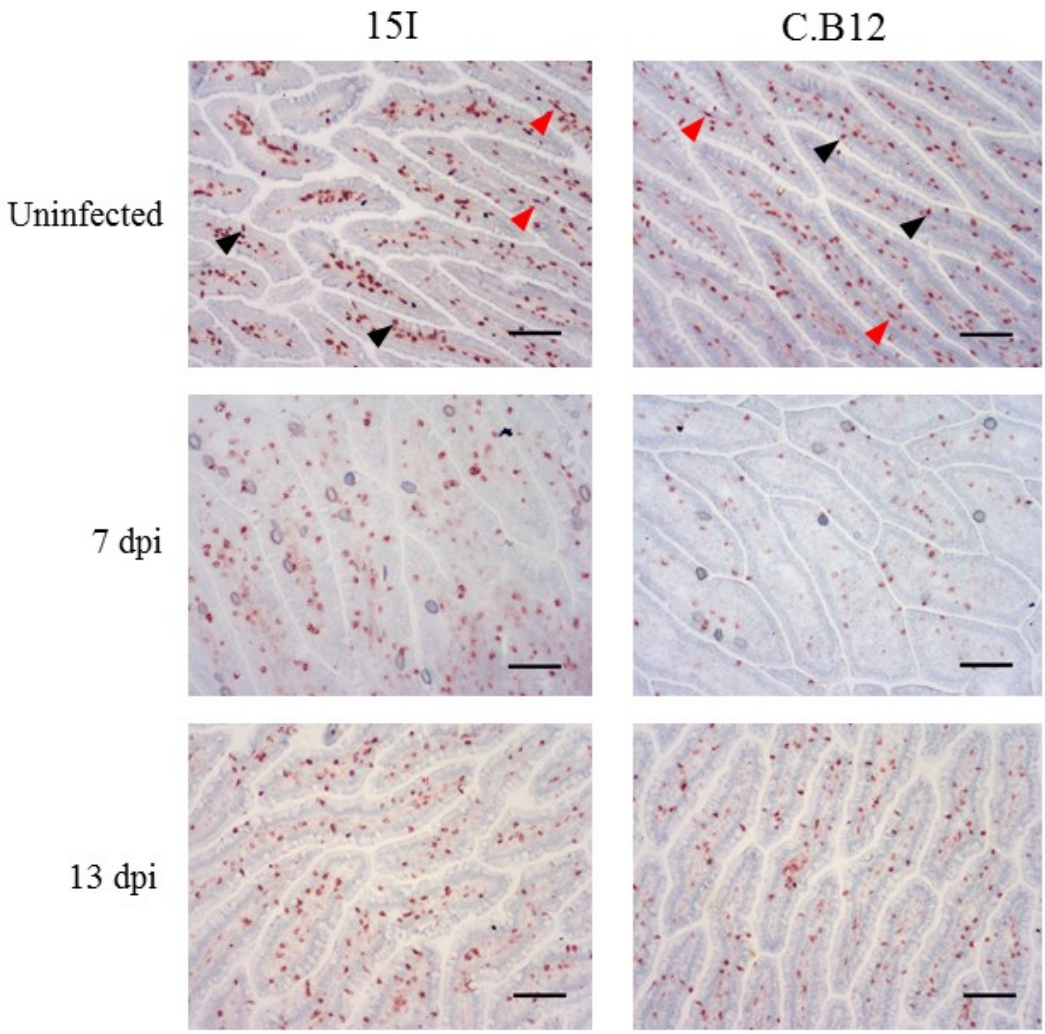

Figure 5-33: CD25$^+$ cells in the villi of the jejunum of *E. maxima*-infected line 15I and C.B12 birds. Three-week-old birds were orally inoculated with 100 *E. maxima* oocysts and samples collected for histology at 7 and 13 dpi. Red AEC stain indicates CD25$^+$ cells. Black arrowheads indicate rounded CD25$^+$ IELs. Red arrowheads indicate CD25$^+$ cells pressed against the basal membrane. Bars represent 100 µm.

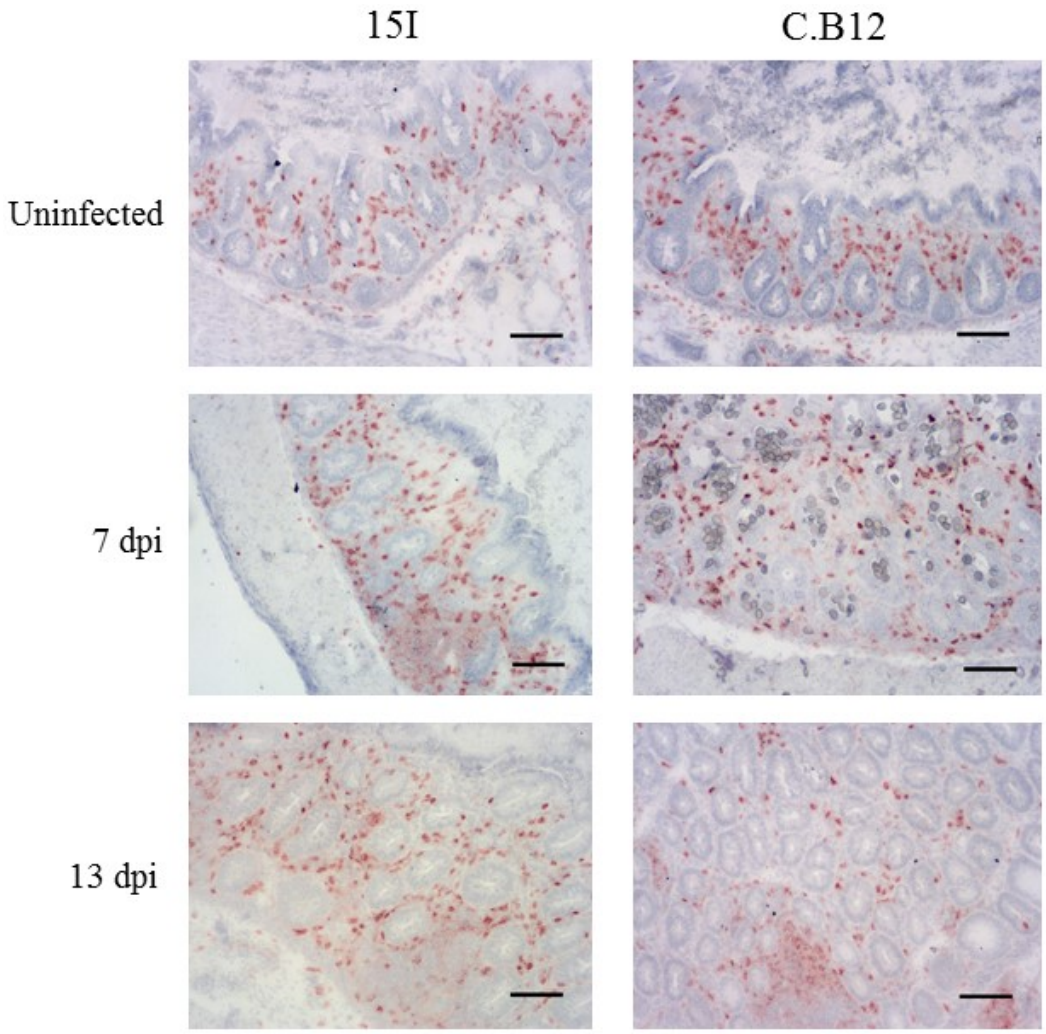

Figure 5-34: CD25$^+$ cells in the caecum of *E. tenella*-infected line 15I and C.B12 birds. Three-week-old birds were orally inoculated with 200 *E. tenella* oocysts and samples collected for histology at 7 and 13 dpi. Red AEC stain indicates CD25$^+$ cells. Bars represent 100 μm.

In the jejunum of control birds of both lines, many chB6$^+$ IELs were present as large cells of mixed morphology throughout both the crypts and villi and are indicative of epithelial NK cells (Vervelde *et al*., 1993). The chB6$^+$ LPL population, indicating B cells, was dependent on the tissue and region of tissue examined, some areas contained very few chB6$^+$ LPLs, whereas other regions contained clusters and areas of higher infiltration of chB6$^+$ LPLs. In addition, follicles with tightly packed chB6$^+$ cells were observed which are germinal centres. High numbers of chB6$^+$ LPLs were observed in both lines in infected and control birds at 7 dpi whereas this was only observed in a few of the infected line C.B12 birds at 13 dpi. In both lines, jejunum chB6$^+$ IELs were increased in the majority of birds of both lines following infection (Figure 5-35, Figure 5-36 and Figure 5-37).

In control caeca, clusters of chB6$^+$ LPLs were observed in all birds, regardless of line or infection status. Few chB6$^+$ IELs were observed in control caeca. Overall there was no change in the chB6$^+$ LPL population following infection, but higher numbers of chB6$^+$ IELs were observed in some of the infected birds of both lines (Figure 5-35 and Figure 5-38).

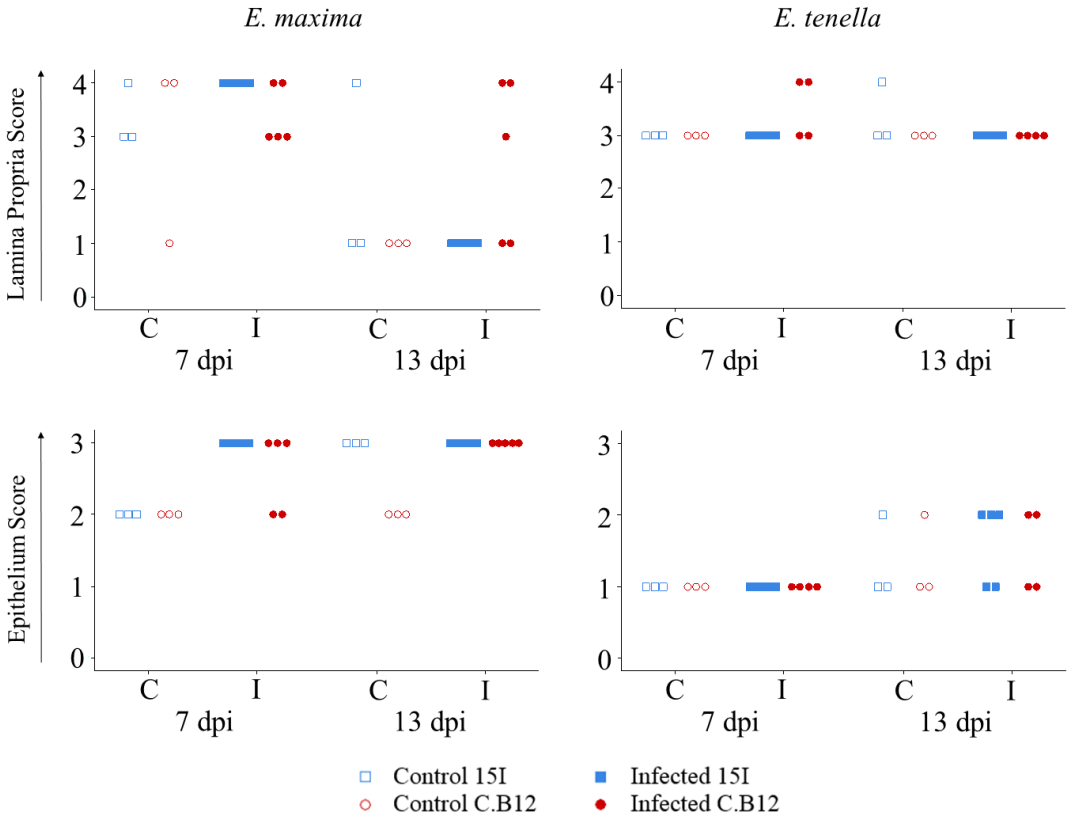

Figure 5-35: ChB6^{+} cells in the jejunum of *E. maxima*-infected and mid-caecum of *E. tenella*-infected lines 15I and C.B12 chickens. Three-week old chicks were orally inoculated with 100 *E. maxima* oocysts, 200 *E. tenella* oocysts or sterile water and samples collected for histology at 7 and 13 dpi. Cell populations were scored as in 2.6.2. Each individual dot represents data from one bird. C; Control. I; Infected.

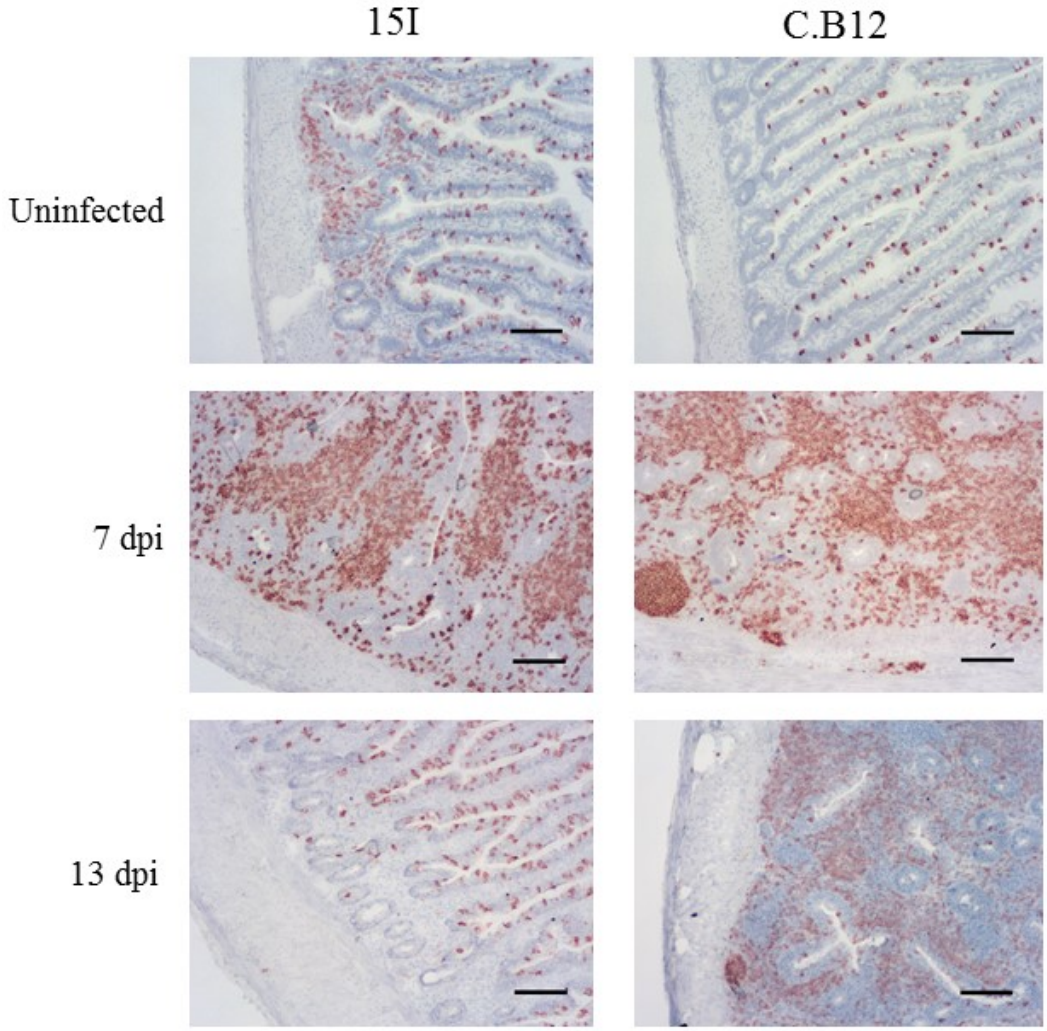

Figure 5-36: ChB6^{+} cells in the crypts of the jejunum of *E. maxima*-infected line 15I and C.B12 birds. Three-week-old birds were orally inoculated with 100 *E. maxima* oocysts and samples collected for histology at 7 and 13 dpi. Red AEC stain indicates ChB6^{+} cells. Bars represent 100 µm.

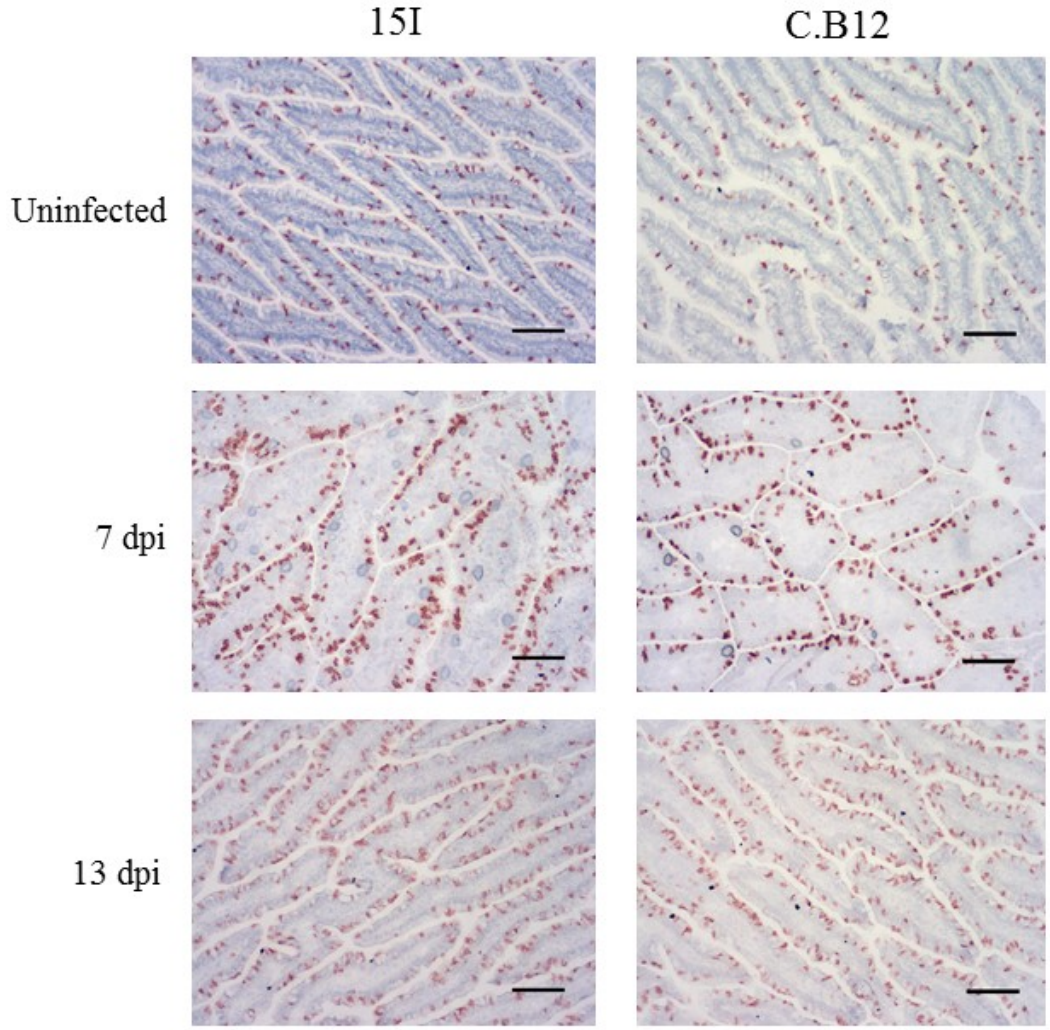

Figure 5-37: ChB6$^+$ cells in the villi of the jejunum of *E. maxima*-infected line 15I and C.B12 birds. Three-week-old birds were orally inoculated with 100 *E. maxima* oocysts and samples collected for histology at 7 and 13 dpi. Red AEC stain indicates ChB6$^+$ cells. Bars represent 100 μm.

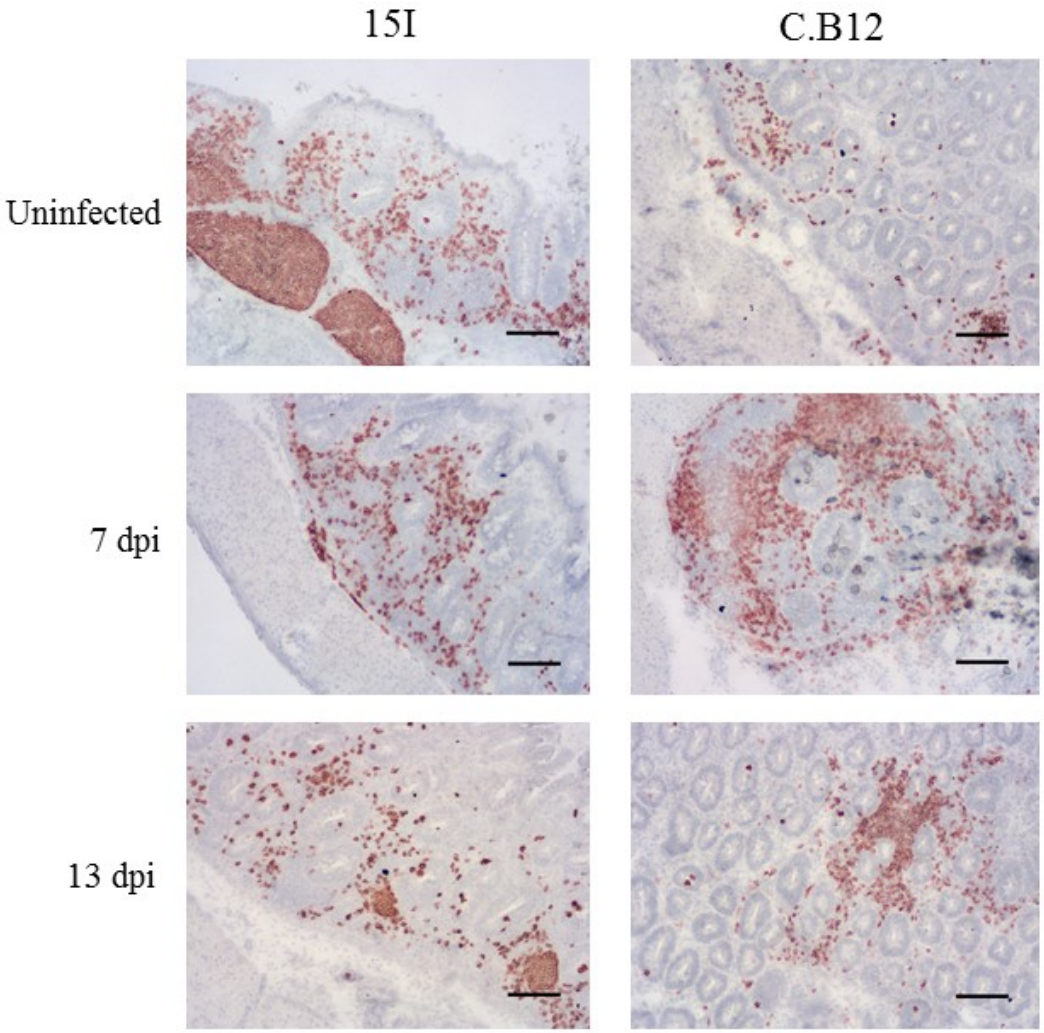

Figure 5-38: ChB6^{+} cells in the caecum of *E. tenella*-infected line 15I and C.B12 birds. Three-week-old birds were orally inoculated with 200 *E. tenella* oocysts and samples collected for histology at 7 and 13 dpi. Red AEC stain indicates ChB6^{+} cells. Bars represent 100 µm.

In jejunum from control 15I and C.B12 birds, MRC1L-B$^+$ (chicken mannose receptor 1, previously described as KUL01$^+$ cells, present on APC) cells were mainly LPLs with very few MRC1L-B$^+$ IELs. MRC1L-B$^+$ cells were of a mixed morphology in both control and infected birds, some were rounded in shape whereas others were more elongated. At 7 dpi with *E. maxima*, some clusters of MRC1L-B$^+$ LPLs were observed in both lines, but these were small in comparison with other cell types and overall no major changes were observed to the number of MRC1L-B$^+$ cells (Figure 5-39, Figure 5-40 and Figure 5-41).

In the caeca of control birds of both lines, the majority of MRC1L-B$^+$ cells were LPLs located just underneath the epithelium and displayed an elongated morphology. Overall no change to the number of MRC1L-B$^+$ LPLs was observed with the exception of a few birds of each line at 13 dpi, where clusters of MRC1L- B$^+$ LPLs were observed. As in the jejunum of *E. maxima*-infected birds, these clusters were far smaller than for other cell types (Figure 5-39 and Figure 5-42).

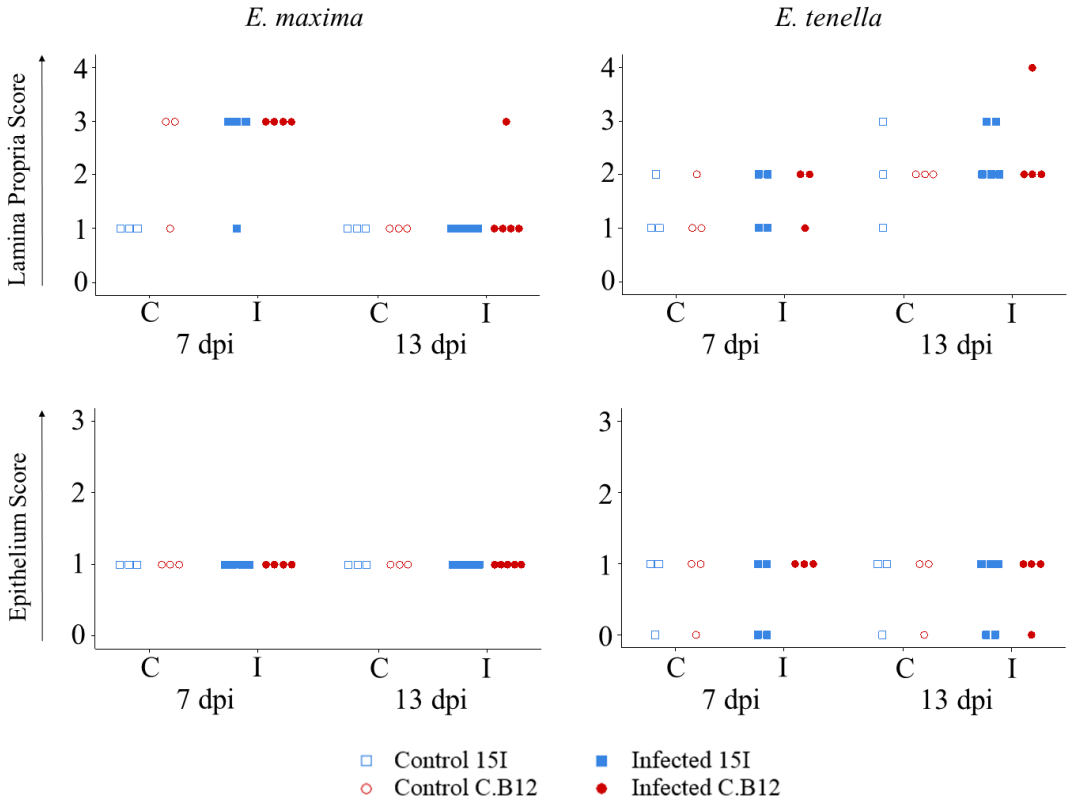

Figure 5-39: MRC1L-B^{+} cells in the jejunum of *E. maxima*-infected and mid-caecum of *E. tenella*-infected lines 15I and C.B12 chickens. Three-week old chicks were orally inoculated with 100 *E. maxima* oocysts, 200 *E. tenella* oocysts or sterile water and samples collected for histology at 7 and 13 dpi. Cell populations were scored as in 2.6.2. Each individual dot represents data from one bird. C; Control. I; Infected.

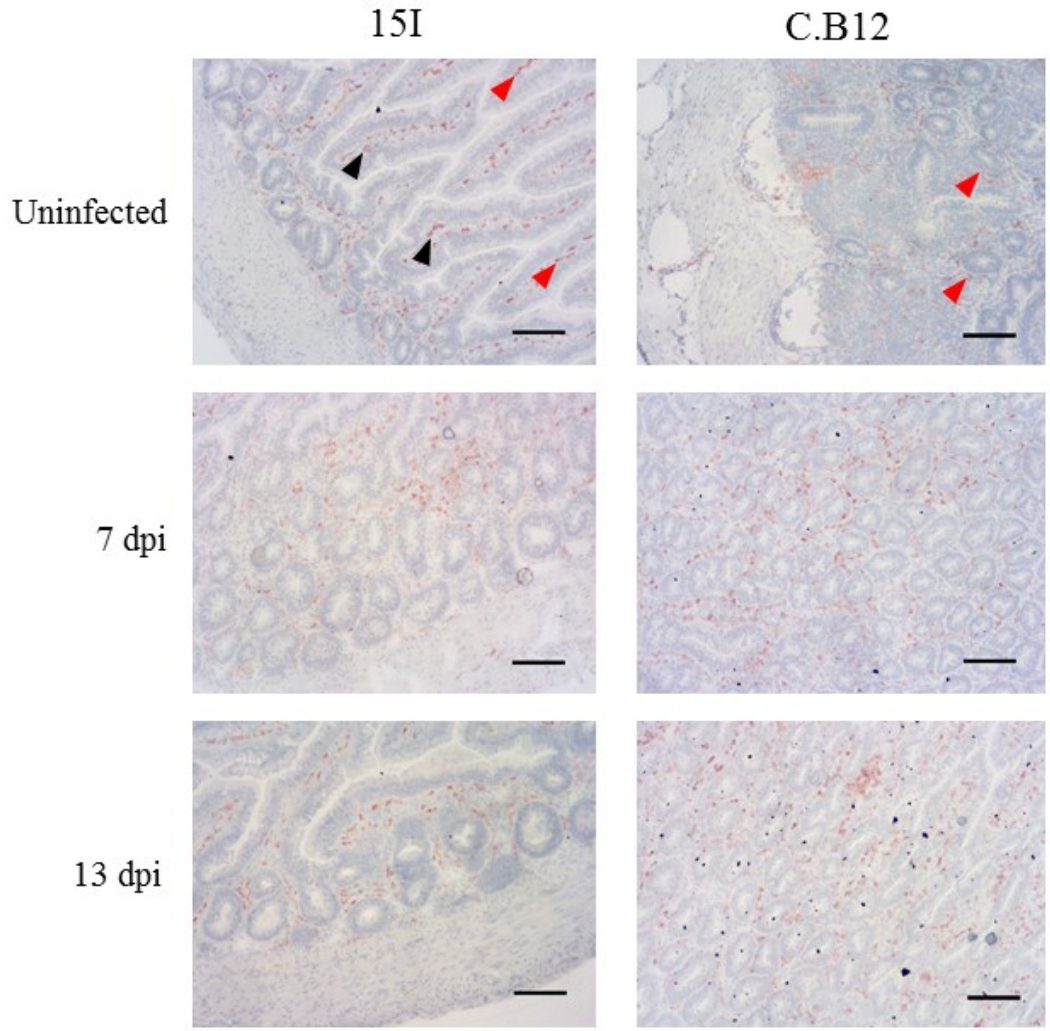

Figure 5-40: MRC1L-B$^+$ cells in the crypts of the jejunum of *E. maxima*-infected line 15I and C.B12 birds. Three-week-old birds were orally inoculated with 100 *E. maxima* oocysts and samples collected for histology at 7 and 13 dpi. Red AEC stain indicates MRC1L-B$^+$ cells. Black and red arrowheads indicate MRC1L-B$^+$ LPLs of rounded and elongated morphology respectively. Bars represent 100 µm.

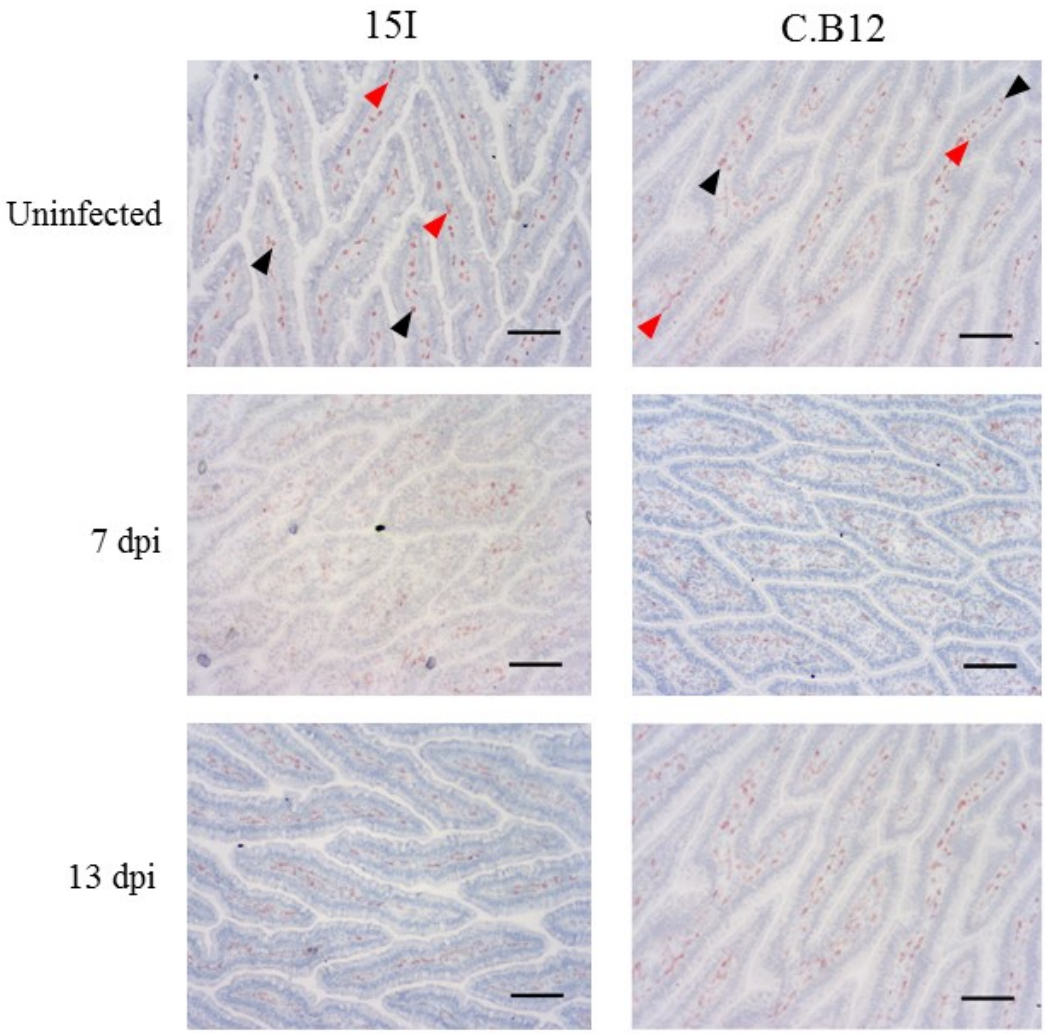

Figure 5-41: MRC1L-B$^+$ cells in the villi of the jejunum of *E. maxima*-infected line 15I and C.B12 birds. Three-week-old birds were orally inoculated with 100 *E. maxima* oocysts and samples collected for histology at 7 and 13 dpi. Red AEC stain indicates MRC1L-B$^+$ cells. Black and red arrowheads indicate MRC1L-B$^+$ LPLs of rounded and elongated morphology respectively. Bars represent 100 µm.

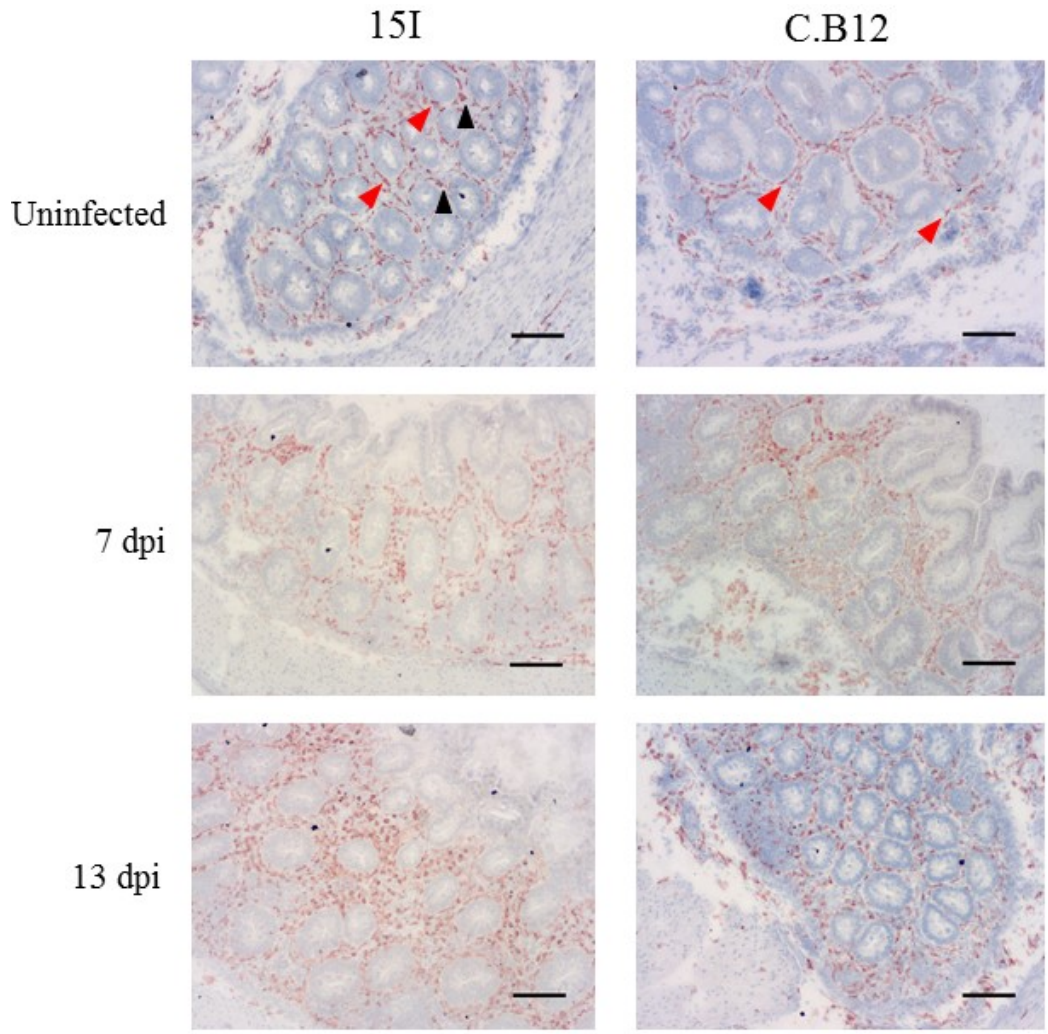

Figure 5-42: MRC1L-B$^+$ cells in the caecum of *E. tenella*-infected line 15I and C.B12 birds. Three-week-old birds were orally inoculated with 200 *E. tenella* oocysts and samples collected for histology at 7 and 13 dpi. Red AEC stain indicates MRC1L-B$^+$ cells. Black and red arrowheads indicate MRC1L-B$^+$ LPLs of rounded and elongated morphology respectively. Bars represent 100 μm.

5.3 Discussion

The aim of this chapter was to test the hypothesis that chicken lines which display differential resistance and susceptibility to *E. maxima* and *E. tenella* infection will exhibit differential immune responses. This chapter aimed to phenotype these differences in order to identify the basis of *Eimeria* resistance. Firstly, to determine resistance and susceptibility to *E. maxima* and *E. tenella*, parasite burden between the two lines was examined. Following *E. maxima* infection, line 15I chickens had significantly higher *E. maxima* load in the jejunum than line C.B12. Given that line 15I is considered susceptible to *E. maxima* in comparison to line C.B12 and 15I chickens produce more oocysts following primary *E. maxima* infection (Bumstead *et al.*, 1995; Smith *et al.*, 2002), it is to be expected that there should be higher parasite burden in the gut of line 15I chickens following infection. On comparison of *E. tenella* load in the caecum of the two lines no differences in copy number were observed. This is in line with previous findings where both lines produce similar numbers of oocysts following primary infection with the Houghton strain of *E. tenella* (Bumstead *et al.*, 1995) and therefore it confirms that no difference in *E. tenella* burden in the caecum following a primary infection was observed between the two chicken lines. However, another study found that following infection with the Weybridge strain of *E. tenella*, line 15I chickens shed fewer oocysts than line C birds following primary infection (Bumstead *et al.*, 1992). Unfortunately, there were not sufficient inbred birds available to study secondary infection in response to the Houghton strain of *E. tenella* and the difference in *E. tenella* burden in the caecum of the two lines following secondary infection could not be examined. However, since line C.B12 chickens produce more *E. tenella* oocysts following

secondary infection than line 15I chickens, line C.B12 would have a higher *E. tenella* genome copy number in the gut than line 15I. Although resistance to the Houghton strain of *E. tenella* is displayed during secondary infection, it is possible that differences in the response to primary infection may explain resistance observed at secondary infection.

Previously, IL-10 was implicated in susceptibility to *E. maxima* and *IL10* mRNA expression was higher at 6 dpi in the jejunum of *E. maxima*-infected line 15I chickens than line C.B12 (Rothwell *et al.*, 2004). Here, chicken lines 15I and C.B12 were infected with *E. maxima* and *E. tenella* and the kinetics of the IL-10 response further characterised at mRNA and protein level, both locally in the gut tissue and systemically in serum. After *E. maxima* infection, increased IL-10 protein and *IL10* mRNA expression was observed in the jejunum of both lines in comparison to control birds. Unexpectedly, localised IL-10 protein levels and *IL10* mRNA expression observed in the jejunum during *E. maxima* infection was higher initially in infected resistant birds up until 4 dpi, but at 5 to 7 dpi it was higher in susceptible birds. Serum IL-10 was also increased in infected birds compared with controls of the same line, but in susceptible line 15I birds, serum IL-10 increased to a much greater extent and for longer in comparison with line C.B12. The increased serum IL-10 levels observed in *E. maxima*-infected line 15I birds indicates that high levels of IL-10 may contribute towards susceptibility to *E. maxima*. However, the level of IL-10 in the serum reflected that of the localised IL-10 response in the jejunum, whereby infected resistant line C.B12 birds had higher serum IL-10 than infected line 15I birds at early time points; but as *E. maxima* infection progressed, this changed and line 15I had higher serum IL-10 than line C.B12. These results indicate that high

levels of IL-10 is a predictor of susceptibility to *E. maxima*, but only from 5 dpi onwards.

Following *E. tenella* infection, serum IL-10 did increase in both lines but overall concentrations were very low and although statistically significant differences were observed, the biological significance of such low concentrations is less convincing, particularly as in many of the birds, serum IL-10 levels fell below the limit of detection of the assay. There was no difference in serum IL-10 between the two lines following *E. tenella* infection and overall *IL10* expression in the caecum did not increase in either line. This is in contrast with a recent study using commercial broilers, which found that serum IL-10 is increased at 5 dpi with *E. tenella* compared to control birds (Wu *et al.*, 2016); however Wu *et al.*, (2016) administered doses of 4 x 10^3 and 35 x 10^3 oocysts to the birds, a much higher dose than in this study. Overall, IL-10 did not appear to be a factor in susceptibility to *E. tenella* however the immune response to a secondary infection with the Houghton strain of *E. tenella* would need to be examined before a role for IL-10 could be ruled out completely.

Other studies have indicated that IL-10 reduces the efficacy of the immune response to *Eimeria.* Antibody-mediated IL-10 depletion in broilers resulted in increased weight gain and decreased oocyst production following inoculation with an attenuated *Eimeria* spp. (*E. maxima*, *E. tenella* and *E. acervulina*) vaccine (Arendt *et al.*, 2016). Moreover, IL-10 depletion had no effect on acquired immunity as chickens treated with anti-IL-10 antibody during vaccination displayed similar weight gains following secondary challenge as chickens that were not treated with anti-IL-10 antibody (Sand *et al.*, 2016). In addition, another study found that in

broilers treated with CitriStim, a yeast mannan feed additive, *IL10* mRNA was reduced in the CT of birds given an attenuated vaccine containing *E. maxima*, *E. tenella* and *E. acervulina* and this was accompanied by reduced oocyst shedding and improved feed efficiency and weight gain (Shanmugasundaram *et al.*, 2013).

Literature surrounding the role of IL-10 during infection with other protozoans is contradictory. In some cases, IL-10 is implicated in increased parasite burden. In *IL10*$^{-/-}$ mice infected with *C. parvum* a reduction in oocyst shedding was observed compared to wild type mice (Campbell *et al.*, 2002). During *T. cruzi* infection, susceptible mice have higher *IL10* mRNA in the spleen (Reed *et al.*, 1994). *IL10*$^{-/-}$ mice display increased resistance through reduced parasite burden in the liver and also exhibit higher IFNγ and NO production compared to wild type mice during *Leishmania donovani* infection (Murphy *et al.*, 2001). However, IL-10 can also be required for limiting immunopathology during protozoan infections. During toxoplasmic encephalitis, *IL10*$^{-/-}$ mice have comparable parasite burden to wild type mice but develop a lethal inflammatory response in the brain characterised by increased influxes of $CD4^+$ T cells, macrophages and proinflammatory cytokines (Gazzinelli *et al.*, 1996; Wilson *et al.*, 2005).

Increased *E. maxima* burden in the gut correlated with increased serum IL-10 and jejunum *IL10* mRNA and is it possible that *E. maxima* is inducing IL-10. In response to recombinant macrophage migration inhibitory factor (MIF)-like protein from *Anisakis simplex* increased *IL10* expression was observed in human PBMCs (Paark *et al.*, 2012). A MIF-like protein has also been identified in *E. acervulina* (Miska *et al.*, 2013) and is potentially involved in enhancing IL-10 production during

Eimeria infection in order to reduce the efficacy of the Th1 response, allowing the parasite to evade the immune response.

Serum IFNγ and jejunum *IFNG* and *IL21* mRNA expression also followed the same pattern as serum IL-10 and *IL10* mRNA expression in the jejunum of *E. maxima* infected birds whereby it was initially higher in infected C.B12 birds early during infection but higher in infected 15I birds at later time points. *IL10*, *IFNG*, and *IL21* expression in the jejunum all correlated positively with one another. As with serum IL-10, serum IFNγ was increased in susceptible line 15I and to a much greater extent and for longer than in line C.B12. These results suggest that the speed at which line C.B12 responds to *E. maxima* infection could at least be partially responsible for the resistance exhibited by this line. It is possible that the earlier production of *IFNG* in the jejunum coupled with the slightly higher expression in uninfected C.B12 birds compared with 15I birds is important in inhibiting parasite replication early on. In *E. vermiformis*-resistant mice, cells taken from the mesenteric lymph node (MLN) were responsive to Con A appear earlier than in susceptible mice and produced more IFNγ (Wakelin *et al.*, 1993) indicating that early timing of the immune response may be important to *Eimeria* resistance. Other studies have indicated that IFNγ is important to resistance to *Eimeria.* In murine studies, mice deficient in IFNγ have increased signs of clinical disease and reduced weight gain compared to mice with intact IFNγ following infection with *E. vermiformis* and *E. pragensis* (Rose *et al.*, 1989). *In vitro* studies have shown that IFNγ is important in reducing the ability of *E. tenella* to replicate within chicken embryo fibroblast and bone marrow macrophage cell cultures (Dimier *et al.*, 1998). It is possible that the higher expression of *IFNG* at early time points is enough to greatly reduce the ability of *E. maxima* to invade and

replicate within host cells, reducing the magnitude of infection. Rothwell *et al.* (2004) found that lines C.B12 and 15I expressed similar levels of *IFNG* at 6 and 9 dpi with *E. maxima.* However in this study, *IFNG* expression was higher in line 15I birds at 6 and 8 dpi. In both studies, birds were given the same dose of *E. maxima* however it should be noted that Rothwell *et al.* (2004) used the Houghton strain whereas the Wisconsin strain was used in this study. To date, infection with these two strains of *E. maxima* have not been directly compared.

In both the jejunum and caecum there were several birds of each line which failed to express *IL21.* Why these birds did not produce *IL21* is unclear. The quality of RNA was checked using an Agilent Technologies 2200 Tapestation and were found to have RNA Integrity Numbers (RIN) of 8 or above. RNA was also re-extracted from these samples and *IL21* was not detected upon repetition of the RT-qPCR. IL-21 is present in the gut of healthy humans and is increased in those suffering Crohn's disease (Monteleone *et al.*, 2005). It seems likely that *IL21* expression should be detected in the gut of all control and infected birds.

Serum IFNγ was increased in both lines in response to *E. tenella* and was increased earlier and to a greater extent in line C.B12 chickens than 15I chickens. *IFNG* and *IL21* in caecal tissue were also increased following *E. tenella* infection, *IL21* slightly later than *IL10* and *IFNG*. Overall no differences were observed between the two lines with *E. tenella* infection which could explain the resistance and susceptibility exhibited upon secondary infection of the two lines. These results indicate that resistance exhibited at secondary infection is not a result of differences in the immune response at primary infection. A possibility is that resistance to *E. tenella*

relies upon the development of an effective memory response and it would be interesting to compare the timing and magnitude of memory B cell and *Eimeria*-specific antibody responses, and the effects of adoptive transfer of lymphocytes on immunity between the two lines.

IFNG expression increased in both resistant and susceptible chicken lines SC and TK respectively after infection with *E. acervulina.* IFNγ was slightly higher in duodenal IELs of susceptible TK chickens at the time of both primary and secondary inoculation but overall no differences were apparent between the two lines (Choi *et al.*, 1999). IFNγ has been associated with disease resistance to protozoan parasites other than *Eimeria*. In *L. donovani* infection in mice, resistant mice have enhanced potency of T cells to produce IFNγ before and during infection whereas susceptible mice have lower T cell production of IFNγ before and during infection (Lehmann *et al.*, 2000). IL-21 is not currently associated with natural host resistance to protozoans however it is required for optimal immune responses to *T. gondii* (Stumhofer *et al.*, 2013b) and *Mycobacterium tuberculosis* infection in mice (Booty *et al.*, 2016).

Overall there was no major changes to *IL2* or *IL6* expression in the jejunum of *E. maxima*-infected birds, although *IL2* expression did mirror that of *IL10*, *IFNG* and *IL21* but this pattern was not as pronounced and *IL2* expression did not correlate quite as strongly with *IL10*, *IFNG* or *IL21*. During *E. vermiformis* infection in mice, resistant mice had higher serum IL-6 compared with susceptible mice and in $IL6^{-/-}$ mice, substantially more oocysts are produced. The same study identified increased serum IL-6 in chickens following *E. tenella* infection although this was not linked to

resistance or susceptibility (Lynagh *et al.*, 2000). Swaggerty *et al.* (2011) also found that broilers that had inherently high levels of proinflammatory mediators including IL-6, CXCLi2 and CCLi2 were more resistant to *E. tenella* infection than chickens with inherently low proinflammatory mediators. There was no inherent difference in *IL6* expression between the two lines observed here and the fairly mild *IL2* and *IL6* response may be due to the low dose of oocysts administered to the birds. In both of the above studies, chickens and mice were challenged with higher doses than in this study and this could account for the lack of IL-6 response observed here.

A relationship between IFNγ, IL-21 and IL-10 has been observed previously. The Smyth line of chickens is considered an animal model for human vitiligo and *IFNG*, *IL21* and *IL10* are upregulated in vitiligo lesions indicating that these cytokines may contribute to the pathogenesis of Smyth line vitiligo (SLV; Shi *et al.* (2012)). During chronic *T. gondii* infection in *IL21*$^{-/-}$ mice, reduced IFNγ-producing CD4^{+} and CD8^{+} T cells are observed in the brain however the number of IL-10 producing CD4^{+} T cells was relatively unaffected indicating that IL-21 is important in promoting the IFNγ response to *T. gondii* but not IL-10 responses (Stumhofer *et al.*, 2013b). In mice during *T. gondii* infection, Th1 cells producing both IFNγ and IL-10 are important to limiting immunopathology (Jankovic *et al.*, 2007) yet these cells prevent successful immunity to *L. major* infection in mice (Anderson *et al.*, 2007). The differences observed could in part be explained by differences in the mechanisms of pathogenicity and sites of replication between the parasites or related to the balance between IFNγ and IL-10 following infection. In human inflammatory bowel disease (IBD), enhanced IL-21 production in the gut contributes to sustained inflammation and is mostly produced by CD4^{+} T cells that also co-express IFNγ (Sarra *et al.*, 2010). However in the case of

human IBD, it appears that IL-21 mediates increased inflammation that results in immunopathology and disease however during *Eimeria* infection, this inflammation could be either beneficial or disadvantageous to clearance of the parasite.

It seems likely that the IL-21 observed during *Eimeria* infection is important to enhancing the innate IFNγ response. IL-21 enhances IFNγ production in human NK and $CD8^+$ T cells (Strengell *et al.*, 2002) and promotes cytotoxicity and IFNγ production in CTLs (Sutherland *et al.*, 2013). During Crohn's disease, IL-12 increases the expression of IL-21 in mucosal explants, and on blocking IL-21, reduced IFNγ production and T-bet expression is observed indicating that IL-21 functions as part of a positive feedback loop thus maintaining the Th1 response (Monteleone *et al.*, 2005). Additionally, IL-21 is also increased during Th2 responses. During ulcerative colitis and *L. major* infection, increased IL-21 is observed coinciding with a Th2 response (Wurster *et al.*, 2002; Fuss *et al.*, 2004). Th17 cells also produce IL-21 and contribute to gut inflammation and the development of severe colitis in mice (Yen *et al.*, 2006). The fact that IL-21 production coincides with Th1, Th2 and Th17 responses indicate that IL-21 may be more important in maintaining T cell responses rather than polarisation of T cells to a particular subtype. IL-21 also functions to attract lymphocytes to sites of inflammation. During ulcerative colitis, IL-21 induces expression of macrophage inflammatory protein 3α (MIP-3α) in epithelial cells (Caruso *et al.*, 2007), an important chemokine for the attraction of lymphocytes. IL-21 also stimulates increased production of MMPs in fibroblasts (Monteleone *et al.*, 2006) for degradation of the extracellular matrix, implying that IL-21 increases tissue damage through inducing the production of MMPs.

IL-21 also has anti-inflammatory functions and has been shown to down-regulate the expression of IL-1β and IL-6 in murine peritoneal macrophages (Li *et al.*, 2013) and production of TNFα, IL-1β, IL-12p70, IFNγ and IL-6 was increased in LPLs of *IL21*$^{-/-}$ mice during DSS-induced gut inflammation (Wang *et al.*, 2016). It is possible that IL-21, while enhancing IFNγ production and T cell responses, reduces the production of proinflammatory cytokines during *Eimeria* infection which may explain the lack of changes to IL-6 expression observed in this study. IL-21 is also shown to elicit immunosuppressive effects through induction of IL-10 production in both TCR-stimulated CD4 and CD8 T cells and Th1 and Th17 polarised T cells (Spolski *et al.*, 2009). In addition, IL-21 stimulated IL-10 production in NK cells *in vitro*, although this was accompanied by increased NK cell cytotoxicity and production of IFNγ as opposed to suppressing NK cell activities (Brady *et al*., 2004).

Following *E. maxima* infection, the main differences in cytokine expression between the lines occurred at early time points and analysis of tissues collected for histology at these time points might reveal phenotypic differences in cell subpopulations in the gut during *E. maxima* infection. However due to timing issues this was not possible here. In general, the cytokine production during *E. tenella* infection was delayed (from 6 dpi) and occurred slightly later than during infection with *E. maxima* (from 4 dpi). The timing of *IL10* production in the caecum following *E. tenella* infection indicates that *IL10* may be important to tissue repair following *E. tenella* infection. The increased *IL10* expression observed following *E. maxima* and *E. tenella* infection is possibly produced by macrophages. Although no changes to the number of macrophages were observed, it is likely that the macrophages become activated during infection and produce IL-10. Late *IFNG* production is likely due to the

presence of a Th1 response as indicated by increased numbers of $CD4^+$ and $TCR\alpha\beta_1^+$ T cells, but may also be produced by the increased $CD8\alpha^+$ cells observed. Early *IFNG* produced is likely by innate cells such as NK cells (increased $chB6^+$ IELs), $TCR\gamma\delta^+$ cells and CTLs. Following *E. maxima* and *E. tenella* infection, the late *IL21* produced is likely involved in the adaptive response to *Eimeria* and could be important to IgG production, increased B cell numbers and GC formation as with *T. gondii* infection (Stumhofer *et al.*, 2013b) and may be being produced by the increased numbers of $CD4^+$, $CD8\alpha^+$ and $TCR\alpha\beta^+$ LPLs or epithelial NK cells ($chB6^+$ IELs) observed. A previous study found that resistant line C chickens exhibited higher numbers of $CD4^+$ and $CD8^+$ PBLs following primary infection than line 15I and higher numbers of $TCR\gamma\delta^+$ PBLs following primary and secondary infection with *E. maxima* but that the numbers of $TCR\alpha\beta^+$ PBLs did not differ between the lines following infection (Bumstead *et al.*, 1995). One study found that in *E. acervulina*-resistant line SC chickens, there were higher numbers of $CD4^+$ IELs in the duodenum following primary infection at early time points, but at later time points, numbers were higher in susceptible line TK chickens. For $TCR\gamma\delta^+$, $TCR\alpha\beta^+$ and $CD8^+$ IELs this trend was reversed although was not as extreme as in the $CD4^+$ IEL population (Choi *et al.*, 1999). The results of this study did not reveal any major differences between the two lines in the subpopulations of these cells in the gut in either infected or control birds. In a study by Swinkels *et al.* (2007), a fast-growing broiler line relatively resistant to *E. acervulina* compared to a slow-growing line had earlier and more pronounced increases in duodenal $CD8\alpha^+$ cells following infection coupled with higher levels of *IFNG* expression indicating that $CD8\alpha^+$ cells are important for resistance and IFNγ production during primary *E. acervulina* infection.

Intra-caecal inoculation of *E. tenella* sporozoites resulted in increased macrophage and $CD4^+$ T cell numbers in the caecal lamina propria within 24 hours post inoculation (hpi) in naive chickens. However, in immune chickens, higher numbers of $CD8^+$ T cells were observed within 8 hpi (Vervelde *et al.*, 1996). Increased granzyme A, FasL and perforin mRNA expression was also observed in the caecum of chickens following secondary *E. tenella* challenge (Wattrang *et al.*, 2016a), indicating that CTL activity is important for protective immunity. In addition, Rose *et al.* (1992) found that $CD8^+$ T cells were important to resistance at secondary infection with *E. vermiformis* and *E. pragensis* in mice. These studies indicate that early increases of $CD8^+$ T cells may be important for effective immunity to *Eimeria*, and it is possible that they increase more rapidly in *Eimeria*-resistant chickens and are responsible for the early production of cytokines following infection compared with susceptible chickens. Early increases in macrophages and $CD4^+$ T cells were observed by Vervelde *et al.* (1996) and these cells may also contribute to early cytokine production observed during *E. maxima* infection in this study. Jeurissen *et al*. (1996) also identified that in *E. tenella* immune chickens, higher numbers of $CD8^+$ T cells are located next to sporozoites in the lamina propria of the caecum compared with naïve birds, and in the caecum of immune birds, numbers of $CD8^+$ T cells increase drastically in the first two days of infection, further implying that $CD8^+$ T cells are important in resistance to *Eimeria.* Breed *et al.* (1997) showed that in *E. tenella*-infected chickens, $CD8^+$ PBLs did not increase until 8 dpi, indicating that initial localised responses are important to resistance.

NK cell activity has also been linked to *Eimeria* resistance. Resistant SC chickens display higher NK cell activity in duodenal IELs at early time points post infection

with *E. acervulina* and *E. maxima* than susceptible FP chickens. However, this study also showed that NK cell activity decreased in the first week of infection before returning to the levels observed in control birds (Lillehoj, 1989). The initial reduction in NK cell activity observed by Lillehoj *et al.* (1989) indicates that NK cells are likely not responsible for the early responses observed in *E. maxima*-resistant birds in this study. However, ICC analysis of tissues from early time points would be required to confirm if this is the case. During *E. vermiformis* infection in mice, resistant mice display higher NK cell activity in the spleen and MLN whereas uninfected susceptible mice have higher resting levels of NK cell activity and are earlier to respond following infection indicating that NK cells are not correlated with resistance in mice (Smith *et al*., 1994).

From this study it appears that the timing and magnitude of the immune response is important in resistance to *E. maxima* and that early production of IFNγ, IL-10 and IL-21 is key to limiting *E. maxima* replication. During *Eimeria* infection, immunity occurs when sporozoites are in transit through the lamina propria, as very few sporozoites reach the crypts (Jeurissen *et al.*, 1996) and fewer schizonts are observed (Vervelde *et al.*, 1995) in immune chickens. It is therefore logical that resistance to *Eimeria* relies on the host response in the first few days of infection, when the majority of sporozoites are present in the lamina propria and in contact with LPLs. It is likely that the exacerbated IL-10 response observed in line 15I is unfavourable and may be limiting the efficacy of the inflammatory and Th1 response. This study was not able to elucidate any potential mechanism of resistance to *E. tenella,* however resistance in line 15I to the Houghton strain of *E. tenella* is apparent following secondary infection (Bumstead *et al.*, 1995) and a follow up trial would be

required to underpin immune mechanisms during secondary infection that may be important to resistance to *E. tenella* in these lines. In conclusion, this study supports high IL-10 levels as a biomarker of susceptibility to *E. maxima*, and identified that early responses during first exposure to the parasite may be a biomarker of resistance. From the time points examined by ICC, it was not possible to determine which cell types might be important for resistance to *E. maxima* and further analysis of tissues collected at earlier time points would be required to determine which cells may be important to initiating early responses.

Chapter 6 Discussion

6.1 Overall perspective

Coccidiosis caused by *Eimeria* is a common intestinal disease in the poultry industry, resulting in reduced weight gain and feed conversion ratio and increased mortality in the birds. Current methods to control *Eimeria*, including the use of anti-coccidial drugs and vaccination, are insufficient. Vaccines are expensive, difficult to produce and require the passage of live *Eimeria* oocysts through the birds whereas *Eimeria* resistance to coccidiostats has been reported (Yadav *et al.*, 2001; Arabkhazaeli *et al.*, 2013). Improved methods to control *Eimeria* in the poultry industry are required. Alternative strategies include the development of live *Eimeria* vaccines expressing *Eimeria* antigens of a different species to elicit protection against multiple *Eimeria* spp. (Marugan-Hernandez *et al.*, 2017), the development of subunit vaccines (Jiang *et al.*, 2012) and the selective breeding of chickens for *Eimeria* resistance. To date, some success has been had in breeding for resistance to *Eimeria* spp. (Rosenberg *et al.*, 1954) showed that it was possible to inoculate chickens with a high dose of *E. tenella* and breed the surviving birds to produce subsequent resistant generations. More recently, (Swaggerty *et al.*, 2015) found that selection of chickens with an inherently high proinflammatory phenotype resulted in increased resistance to *E. tenella.* However, resistance to one spp. of *Eimeria* does not result in resistance to another and it is of interest to the poultry industry to identify biomarkers of resistance to multiple *Eimeria* spp., so that these biomarkers can be used for selection of chickens resistant to multiple *Eimeria* strains within commercial broiler populations. The development of subunit vaccines and identification of resistance biomarkers both require prior knowledge of the chicken immune response to *Eimeria.* This study aimed to further characterise innate and Th17 responses during

Eimeria infection and identify biomarkers of *Eimeria* resistance through characterisation of the immune response of different chicken lines which differ in their resistance and susceptibility to different *Eimeria* spp.

One of the main aims of this study was to investigate the responses of APC to *Eimeria* antigens including vaccine candidates IMP1 and AMA1 and to identify which chicken PRRs recognise these antigens. The results of this study support the hypothesis that APC promote inflammation, Th1 and regulatory responses during *Eimeria* infection and vaccination with IMP1 and AMA1, and that IMP1 and AMA1 are recognised by TLRs on the APC surface. The ability of IMP1 and AMA1 to be recognised by chicken TLRs and initiate responses in APC support their potential for use as subunit vaccines, which would provide an alternative to the use of live oocysts.

Eimeria infection elicits a Th1 response, but less is known about Th17 responses during infection. In this study, Th17 responses were hypothesised to contribute to immunopathology during *Eimeria* infection, and consequently are a target to improve the overall outcome of *Eimeria* infection in chickens. *E. maxima* and *E. tenella* infection of commercial broilers indicated that Th17 responses are not involved in immunity to *Eimeria*, either in contribution to immunopathology or in the resolution of infection. Although Th17 responses were not involved, IL-21 was identified as a novel cytokine involved in the response to *Eimeria,* but it is not yet clear if IL-21 is beneficial or harmful to clearance of the parasite, and whether it can be targeted to improve the outcome of *Eimeria* infection.

Selective breeding of chickens resistant to *Eimeria* infection represents an attractive strategy to combat coccidiosis in the poultry industry, however biomarkers of resistance are required for selection of resistant chickens. Rothwell *et al*. (2004) showed that *IL10* mRNA expression was higher in the jejunum of *E. maxima*-susceptible chickens compared with resistant chickens. As these two chicken lines exhibit differential resistance to *Eimeria*, it is expected they will exhibit differential immune responses during infection, and higher IL-10 in the serum and gut will result in susceptibility to *Eimeria*. This study aimed to identify biomarkers of *Eimeria* resistance through phenotyping the immune response to *E. maxima* and *E. tenella* in these two lines. This study confirmed that high levels of gut and serum IL-10 is a marker of susceptibility to *E. maxima*, but only at later time points. This study also identified that early immune responses in the two lines differ and may be indicative of resistance to *E. maxima*, providing a basis from which to identify biomarkers of *Eimeria* resistance in commercial populations of chickens.

6.1.1 Chicken APC responses to *Eimeria* antigens

The current literature implies that APC drive inflammatory processes by increasing the production of proinflammatory cytokines during *Eimeria* infection, however these studies have relied heavily upon the use of macrophage-like cell lines. The development of BMM (Garcia-Morales *et al.*, 2014) and BMDC (Wu *et al.*, 2010) primary cell cultures from chicken bone marrow cells present the opportunity to study intrinsic responses of primary cells to antigens. In line with current literature, chicken BMM and BMDC increased expression of *IL1B*, *IL6* and *NOS2* in response to crude sonicated *E. tenella* oocysts (EtAg), indicating macrophages and DCs facilitate inflammation during *Eimeria* infection. The results presented in this study

suggest that DCs could be important producers of IFNγ during the innate phase of the immune response to *Eimeria* and in promoting Th1 responses as observed during infection with other protozoan parasites such as *L. major* (Martínez-López *et al.*, 2015). Although EtAg stimulation did not alter *IFNG* expression, BMM did express *IFNG* and indicating that macrophages can produce IFNγ during *Eimeria* infection. Innate IFNγ produced by DCs, macrophages and NK cells, is likely important to enhancing macrophage activation through increased phagocytosis and inflammatory cytokine and iNOS production, therefore reducing *Eimeria* replication. BMM responded to EtAg in a regulatory fashion by upregulating *IL10* expression, implying that macrophages could be a producer of IL-10 during the early stages of *Eimeria* infection. In mice, IL-10, produced by intestinal macrophages, is important to preventing excessive innate responses to the *Citrobacter rodentium* bacterium by limiting IL-23 production (Krause *et al.*, 2015). Furthermore, IL-10 is required for mucosal homeostasis and loss of IL-10 signalling results in impaired generation of intestinal anti-inflammatory macrophages, leading to severe colitis (Shouval *et al.*, 2014).

Th17-associated cytokines are also important to innate immunity and can induce IL-1β and TNFα production by human macrophages (Jovanovic *et al.*, 1998) and in murine tissue- and bone marrow-derived macrophages, IL-17A induces IL-12 and IL-17A production, although this can be suppressed by IL-10 (Gu *et al.*, 2008). Innate IL-17A can also influence macrophage function; IL-17A recruits macrophages to sites of inflammation, induces IL-12p70 expression and enhances antigen presentation in murine macrophages (Barin *et al.*, 2012). This study investigated the effects of direct EtAg stimulation of APC, but does not account for the cytokine

milieu present during inflammation *in vivo* and the presence of other innate cell types and responses. To investigate the effects of external cytokines on the influence of macrophage and DC function, addition of recombinant cytokines, such as IL-17A and IL-10, to cultures would examine the effects these cytokines exert on APC activity in response to *Eimeria*. To investigate the role of macrophages and DCs in relation to resistance and susceptibility, an important step would be to characterise the responses to *Eimeria* antigens of BMM and BMDC cultured from resistant and susceptible chicken lines. This would determine if resistance lies in the efficiency of APC to produce higher levels of proinflammatory cytokines or more efficiently stimulate Th responses. It would also be of interest to study the response of BMM and BMDC from resistant and susceptible chickens to live and heat-killed sporozoites to determine if APC from resistant chickens are better able to phagocytose and kill sporozoites, or if APC from susceptible chickens are more prone to invasion by sporozoites.

Success in establishing protective immunity to *Eimeria* by using recombinant *Eimeria* proteins and DNA vaccines has been reported (Yin *et al.*, 2013; Hoan *et al.*, 2014) however the role of APC in the development of protection has not been fully assessed. This study confirmed that BMM did have the capacity to elicit inflammatory responses to IMP1 and AMA1, but did not necessarily do so to antigens from both *E. maxima* and *E. tenella*. This result is, in part likely due to there being insufficient quantities of protein in the supernatants and the likely overestimate of the concentrations present. To improve upon these results, it would be necessary to purify the recombinant proteins from the cell supernatant and quantify the concentrations using other methods such as NanoDrop^{TM} technology or

by Bradford Assay. Thereafter, the ability of BMM to initiate inflammatory and Th responses could be interrogated with greater confidence.

Based on morphology, BMM and BMDC cultures are highly heterogeneous and cells between the cultures share morphological characteristics indicating that there is some overlap in the cell populations of the two. In order to validate BMM and BMDC cultures as suitable tools for studying chicken macrophage and DC responses to antigens it is important to understand the phenotypic differences between the two cultures and to confirm if these cultures do possess properties typical of tissue DCs and macrophages, work which is currently ongoing in the Vervelde lab (Roslin Institute, UK).

6.1.2 Host recognition of *Eimeria*

Currently it is unknown which *Eimeria* PAMPs are recognised by which chicken PRRs. This study concludes that IMP1 from *E. tenella* and AMA1 from *E. maxima* and *E. tenella* are recognised by chicken TLR1B/2A heterodimers. In both mammals and chickens, the TLR1/2 family recognise bacterial lipoproteins and peptidoglycans however the ability of this family to form heterodimers likely allows for an increased capacity to recognise a wider range of antigens. The SEAP reporter gene assay is currently developed for TLR1B/2A and TLR1B/2B heterodimers and TLR21, and is under development for TLR5, TLR4 and TLR15. Common TLR agonists amongst other protozoan parasites, GPI anchors from *Plasmodium*, *Leishmania*, *Toxoplasma* and *Trypanosoma* appear to be recognised by mammalian TLR2 in heterodimers with TLR1, 6 or 4, dependent on the genus of parasite (Ghosh *et al.*, 2013). Therefore, *Eimeria* GPI anchors represent likely candidates for recognition by heterodimers formed between chicken TLR1A and B and TLR2A and B.

Additionally, *T. gondii* profilin is recognised by murine TLR11 (Yarovinsky *et al.*, 2005), which can also recognise bacterial flagellin (Mathur *et al.*, 2012). While TLR11 has not been identified in the chicken, TLR5, which recognises bacterial flagellin, is a candidate receptor for the recognition of *Eimeria* profilin. Other PRRs also exist out with the TLR family, including C-type lectins (CLRs), RIG-I-like receptors (RLRs) and Nod-like receptors (NLRs) and it is probable these PRRs are involved in recognition of *Eimeria* PAMPs. Development of the assay for these PRRs would allow for further investigation and as alternatives to reporter gene assays, other techniques, such as ligand binding assays that make use of radio or fluorescently labelled ligands, could be used. *Eimeria* also express a wide range of immunogenic surface antigens (Chow *et al.*, 2011) and micronemes (Huang *et al.*, 2015; Hoan *et al.*, 2016) involved in host cell invasion and it would be interesting to determine which TLRs could recognise these *Eimeria* antigens, particularly as they are at the interface of host-parasite interaction. Knowledge regarding which *Eimeria* PAMPs are recognised by which chicken PRRs provides insight into the signalling pathways required for successful immunity to *Eimeria* and vaccination, presenting opportunities for the selection of vaccines based on their ability to stimulate the required response, and identify suitable vaccine adjuvants to enhance these responses thereby increasing vaccine efficacy. Furthermore, it would be interesting to compare polymorphisms in the PRRs of resistant and susceptible chicken lines, and to determine whether PRRs from resistant chickens are more efficient at recognising *Eimeria* PAMPs and therefore in initiating downstream responses.

6.1.3 Th17 responses during *Eimeria* infection

The fact that IL-17A-D, IL-17F, IL-21, IL-22, IL-23, IL-23R genes and a partial sequence for the Th17 transcription factor RORγt have been identified in the chicken genome (Kaiser *et al.*, 2005; Kim, S. *et al.*, 2012; Rothwell *et al.*, 2012; Welch, 2015) and chicken $CD4^+$ splenocytes expressing IL-17A and IL-17F have been detected (Walliser *et al.*, 2017) provides compelling evidence for the existence of Th17 responses in the chicken. Th17 responses are thought to contribute to immunopathology during *Eimeria* infection. During *E. falciformis* infection in $IFN\gamma^{-/-}$ mice, Th17 responses were shown to both contribute to increased immunopathology but also limit parasite burden (Stange *et al.*, 2012). In chickens, antibody-mediated depletion of IL-17A reduced immunopathology during *E. tenella* infection (Zhang *et al.*, 2013). In the present study, no changes in the expression of Th17-associated cytokines, with the exception of IL-21, was observed indicating that Th17 responses did not enhance immunopathology or limit parasite burden in these birds. This is contradictory to previous reports where increased IL-17A and IL-17F have been observed following *E. maxima* and *E. tenella* infection (Hong *et al.*, 2006a; Kim, W. *et al.*, 2012). IL-21 is not currently associated with *Eimeria* infection, and presents a novel cytokine expressed during *Eimeria* infection. Although considered an effector and inducer of Th17 responses, IL-21 is highly pleiotropic in nature and further investigation is required to decipher its importance during *Eimeria* infection and its potential as a therapeutic target. It would be interesting to investigate responses to *Eimeria* in *IL21*$^{-/-}$ chickens, to determine its importance to NK and $CD8^+$ T cell cytotoxicity, $CD4^+$ T cell responses, *Eimeria*-

specific antibody production and the development of immune memory and protection, but also to the overall effect on parasite replication following infection.

6.1.4 Biomarkers of resistance to *Eimeria*

Following infection with protozoan parasites, IL-10 can be a factor in host susceptibility (Reed *et al.*, 1994; Campbell *et al.*, 2002) or in limiting immunopathology (Gazzinelli *et al.*, 1996; Wilson *et al.*, 2005). IL-10 is associated with host susceptibility to *Eimeria* and it was previously shown that *E. maxima* susceptible line 15I exhibited higher gut *IL10* expression than resistant line C.B12 following infection (Rothwell *et al.*, 2004). In support of Rothwell's findings, gut *IL10* mRNA was generally higher in *E. maxima*-infected line 15I (susceptible) chickens than line C.B12 (resistant) chickens and serum IL-10 was increased to a much greater extent in susceptible line 15I than in line C.B12. These results indicate IL-10 is potentially important in limiting immunopathology but when excessive levels are produced, as observed in line 15I, IL-10 is detrimental to clearance of the parasite and susceptibility ensues.

This study is novel in that it investigated the kinetics of the innate immune response of two lines differing in their resistance to *E. maxima* and *E. tenella*. While the study by Rothwell *et al*. (2004) found that IL-10 mRNA in the gut was higher in *E. maxima*-susceptible chickens during infection, it did not investigate the kinetics of early and late responses between the two lines. A main finding of the present experiment was that at early time points (2 and 4 dpi), *IFNG*, *IL10* and *IL21* mRNA expression was higher in the gut of infected resistant line C.B12 birds than in line 15I, but at later time points (5 to 8 dpi), expression was higher in infected line 15I

birds than line C.B12 suggesting that the timing of the immune response is an important factor in resistance to *Eimeria*, and early cytokine production is key to resistance following primary infection with *E. maxima*. In addition, measuring IL-10 as a biomarker for susceptibility to *E. maxima* is only valid at later time points. A previous finding in murine *Eimeria* infection where cells taken from the mesenteric lymph nodes of *E. vermiformis*-resistant mice produce IFNγ earlier in response to stimulation with ConA or parasite antigen than susceptible mice supports this study in that rapid responses are important to resistance (Wakelin *et al.*, 1993).

Overall, very little difference was observed between the two lines following *E. tenella* infection and investigation of the responses of the two lines following a secondary *E. tenella* infection is warranted, as secondary infection is when differential resistance between these lines is exhibited (Bumstead *et al.*, 1995). However, *IL10* mRNA and protein in the caecum was higher at later time points (6 and 7 dpi) in *E. tenella*-susceptible line C.B12 birds compared with resistant line 15I, as was *IFNG* mRNA in the caecum and serum IFNγ. Although not proven, this could indicate that higher IL-10 and IFNγ may be biomarkers of susceptibility to *E. tenella*. To confirm this, it would be necessary to determine if higher IL-10 and IFNγ correlated with susceptibility during secondary *E. tenella* infection, as this is when resistance and susceptibility to *E. tenella* is demonstrated. Unfortunately, due to low availability of the inbred lines, it was not possible to proceed with a secondary infection in this study. Due to the low levels of IL-10 and IFNγ in the sera of uninfected birds, serum IL-10 and IFNγ levels are unlikely to be suitable predictors of *Eimeria* resistance prior to infection.

6.1.5 IFNγ, IL-10 and IL-21 during *Eimeria* infection

An overview of the cells likely producing IFNγ, IL-10 and IL-21 and the effector functions of these cytokines is given in Figure 6-1. The role of IFNγ may be to inhibit parasite replication within the host, likely through enhancing inflammation and the phagocytic capacity of macrophages, and enhancing NK cell and CTL cytotoxicity and IFNγ production. IL-10 and IL-21 are both pluripotent cytokines, produced by multiple cell types, and potentially fulfil multiple roles during *Eimeria* infection. Although primarily considered a Th17 cytokine, IL-21 is also produced by $CD8^+$ T, B and NK cells and is important for optimum Th1, memory and humoral responses during *T. gondii* infection (Stumhofer *et al.*, 2013b). Given the positive correlation between IL-21 and IFNγ in this study, it could be that IL-21 is involved in promoting IFNγ and Th1 responses. IL-21 has been shown to induce IFNγ production in human primary NK and T cells (Strengell *et al.*, 2002) and, during antigen presentation to NKT cells in mice, IL-21 stimulation of DCs results in increased IFNγ production by the NKT cells (Maeda *et al.*, 2007). The innate IL-21 observed in this study up to 5 dpi is likely involved in inducing IFNγ production in innate cells, such as NK cells, during *Eimeria* infection whereas IL-21 produced at later time points more likely contributes to initiating and maintaining Th1 responses and the responses of $CD8^+$ T lymphocytes, correlating with enhanced numbers of $chB6^+$ and $CD8\alpha^+$ IELs (NK cells) and $CD4^+$ and $CD8^+$ LPLs (T cells and CTLs) in infected Ross 308 broilers (Chapter 4). Alternatively, IL-21 may also be involved in regulating the inflammatory response. In murine LPS-stimulated peritoneal macrophages, IL-21 reduced production of IL-1β, TNFα and IL-6 but did not affect IFNγ and IL-10 production (Li *et al.*, 2013) and inhibits DC maturation and activation (Brandt *et al.*, 2003). During infection of the inbred lines (Chapter 5),

IL21 mRNA was increased following infection however *IL6* was unchanged. It is therefore possible that IL-21 down-regulated IL-6 production in these birds following *Eimeria* infection.

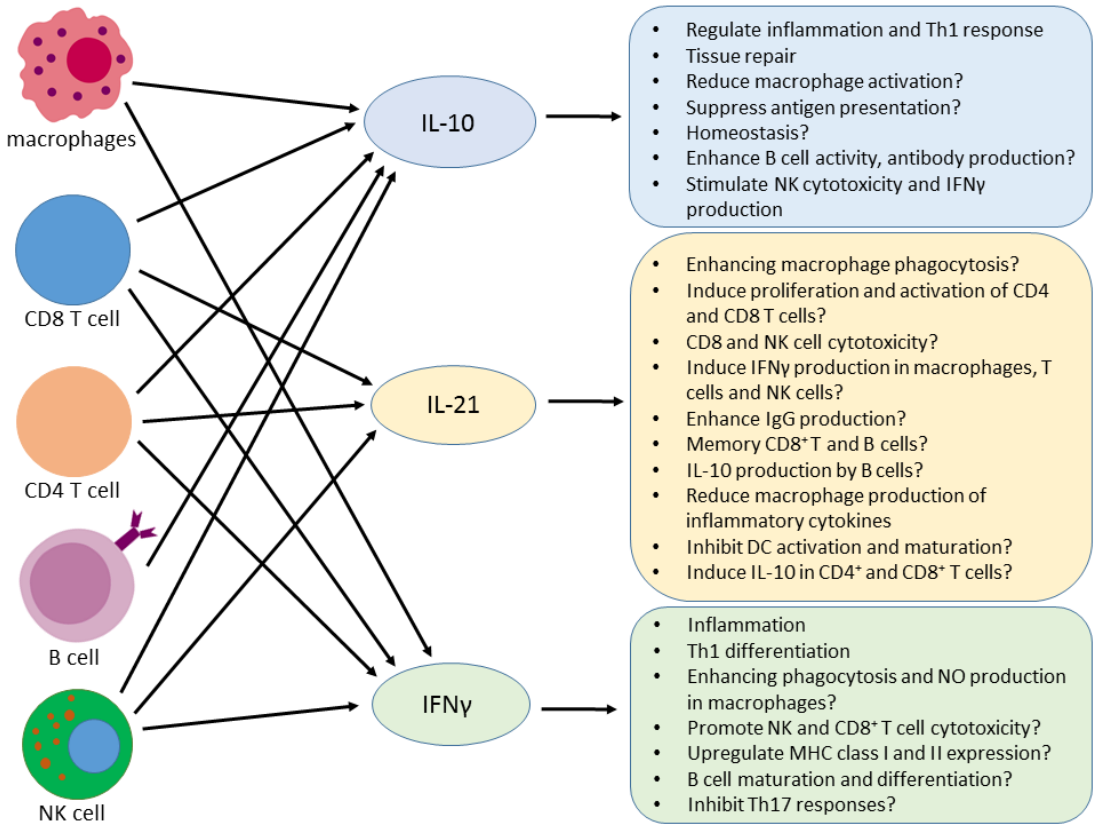

Figure 6-1: IFNγ, IL-10 and IL-21. Overview of the cell types that could be producing IFNγ, IL-10 and IL-21 and the effector functions of these cytokines during *Eimeria* infection.

As Th1 responses are required for successful immunity to the *Eimeria* parasite, it follows that IL-10 instigates susceptibility through limiting the efficacy of the Th1 response. However, IL-10 also correlates positively with IFNγ and IL-21 indicating that it may, to a degree, be important in limiting immunopathology. In addition, IL-10 also holds immuno-stimulatory properties which include enhancing proliferation

and Ig production in B cells (Rousset *et al.*, 1992), enhancing proliferation of mast cells (Thompson-Snipes *et al.*, 1991) and when combined with IL-18, enhancing NK cell cytotoxic activity and production of IFNγ (Cai *et al.*, 1999).

The relationship between IFNγ, IL-21 and IL-10 has been demonstrated elsewhere. In the Smyth line of chickens, an animal model for human vitiligo, IFNγ, IL-21 and IL-10 are co-expressed and coincide with a predominance of B and $CD8^+$ T cells observed in vitiligo lesions (Shi *et al.*, 2012). In human systemic lupus erythematosus (SLE), IL-21 induces IL-10 production in activated $CD4^+$ and $CD8^+$ T cells (Spolski *et al.*, 2009) and IFNγ production in $CD8^+$ T cells during chronic lymphocytic choriomeningitis virus (LCMV) infection (Elsaesser *et al.*, 2009; Fröhlich *et al.*, 2009; Yi *et al.*, 2009). IL-21 is increased in the gut during human IBD and is produced by $CD4^+$ T cells that also co-produce IFNγ (Sarra *et al.*, 2010). Additionally, Th1 cells that co-produce IL-10 are also involved in other protozoan infections. During *T. gondii* infection, IL-10 important to limiting immunopathology, is produced by Th1 cells that also co-express IFNγ and is thought to be a self-limiting mechanism of the Th1 response (Jankovic *et al.*, 2007). In contrast, during *L. major* infection, IL-10 produced by IFNγ-producing Th1 cells prevents complete clearance of the parasite (Anderson *et al.*, 2007).

6.1.6 General considerations and limitations

Initially the IL-10 (1) primers and probe (Chapter 2 as published by Rothwell *et al.* (2004)) were used to measure IL-10 expression in BMM and BMDC cultures. When measured in control Ross 308, line 15I or line C.B12 tissues, little or no *IL10* mRNA was detectable, in line with the levels published by Rothwell *et al.* (2004). However, it was observed that this primer and probe set returned unreproducible curves across

triplicate wells. As IL-10 helps to maintain gut homeostasis (Mantovani *et al.*, 2014), it was expected that *IL10* should be expressed in the gut of control birds. The primers and probe were redesigned (*IL10* (2)) and upon repetition of the RT-qPCR, *IL10* was found to be expressed in tissues from both control and infected birds in both Ross 308 broilers and lines 15I and C.B12. Expression of IL-10 in control tissues of other chicken lines was also assessed independently by other lab members using the EvaGreen® qPCR, employing a different primer set to those used in this study, and was found to be expressed at similar levels as observed in this trial, implying that *IL10* mRNA is expressed in the gut under normal homeostatic conditions.

The use of a scoring system to analyse changes to the cell sub-populations in the gut following *Eimeria* infection was decided upon as many cell clusters were observed throughout the gut and it was expected that these would lie close to the *Eimeria* parasite. During *Eimeria* infection of Ross 308 broilers, this provided a suitable method to determine the general picture of how cell sub-populations changed following *Eimeria* infection and provided indications as to which cells were expressing the increased *IFNG*, *IL10* and *IL21* mRNA observed. However, during *Eimeria* infection of the inbred lines, the use of the scoring system did not allow for detection of subtle differences between the lines and no apparent differences were discernible, in either control or infected birds. To improve upon this and provide quantitative data on differences between the two lines, an automated method to analyse stained tissues to determine absolute numbers of cells present should be used. However, due to time constraints, this was not possible in this study.

Alternatively, at the time of tissue collection, the IEL and LPL sub-populations could be analysed by FACS.

Infection of *Eimeria* susceptible and resistant inbred lines revealed that an early immune response appears essential for resistance to *E. maxima*. It would be necessary to perform histological analysis of tissues collected at earlier time points from *E. maxima*–infected inbred lines in order to associate which cell types are important in implementing the sharp increase in *IFNG*, *IL10* and *IL21* observed in resistant line C.B12 compared with that of line 15I.

6.2 Future work

The advent of gene editing technology has simplified the production of knock out animals. Use of *IL10* knockout chickens would determine if increased IFNγ and inflammatory responses were observed during *Eimeria* infection in the absence of IL-10, and if this resulted in reduced parasite burden or increased immunopathology. IL-21-deficient chickens would allow examination of IL-21 activities during *Eimeria* infection and would determine whether it is responsible for enhancing IFNγ-mediated cellular responses and humoral immunity as during *T. gondii* infection (Stumhofer *et al.*, 2013b). The use of *IL10* and *IL21* knock out chickens would also define the influence that these cytokines have upon the production of one another and IFNγ. In addition, it would be interesting to compare the level of immunopathology and parasite burden in chickens lacking in Th17-associated cytokines with their wild type counterparts to determine the contribution of Th17 cells to immunopathology or resolution of infection.

The early timing of the immune response appears to be fundamental for resistance to *E. maxima* however, as expected, no phenotypes were identified relating to resistance or susceptibility to primary infection with *E. tenella*. RNA sequencing (RNA-Seq) of the gut tissue of the inbred lines would provide a wider overview of the immune response and possibly identify further differences in the immune phenotypes of the two lines. Analysis of the phenotypic variances between the two inbred lines in response to a secondary *E. tenella* infection would also likely highlight potential biomarkers of resistance, as resistance in these lines is not displayed until secondary challenge with the Houghton strain of *E. tenella* as used in this study (Bumstead *et al.*, 1995).

The degree of severity of *Eimeria* infection is dependent upon multiple factors including the genotype of the host, the species and strain of *Eimeria*, the number of oocysts administered and previous exposure to *Eimeria*. Here the responses of resistant versus susceptible chickens to *E. maxima* and *E. tenella* were compared but the influence of variation between different *Eimeria* spp. and strains on the final outcome was not evaluated. It is a possibility that resistance and susceptibility is partially dependent on the ability of *Eimeria* to recognise, infect and replicate within different hosts. It is not yet exactly clear how each *Eimeria* spp. recognises its preferred site of infection and resistance observed in some chicken lines could in part be due to a varying ability of *Eimeria* spp. to recognise and invade particular hosts thus influencing disease outcomes. As already developed in the mouse (Sato *et al.*, 2009), the development of chicken gut organoid cultures will also present an attractive opportunity to study the interaction between sporozoites and host cells *in*

vitro, and to study if sporozoites can invade and replicate more efficiently in organoid cultures from susceptible hosts.

6.3 Conclusion

Understanding the immunological basis of resistance to *Eimeria* is of particular interest to the poultry industry. With increasing pressure to deliver food security and improve the welfare of livestock, selectively breeding animals for disease resistance provides an attractive proposition. This study identified IL-21 as a novel cytokine in the response to *Eimeria* infection, however its function, whether beneficial or harmful, is unclear and further study is required to determine its suitability as a target to improve the outcome of infection or as a biomarker of resistance. The results of this study show that a rapid (up until 4 dpi) increase in jejunum IFNγ, IL-10 and IL-21 mRNA production is related to resistance to *E. maxima* whereas at 6 and 7 dpi, an increase in IL-10 production in the serum and gut is related to susceptibility to *E. maxima*. This indicates that high levels of IL-10 in the serum and gut are biomarkers of susceptibility to *E. maxima* at later time points. From infection of the inbred lines it was evident that a relationship exists between IFNγ, IL-21 and IL-10 responses to *Eimeria*. It is possible that maintenance of the correct balance of these cytokines is crucial to avoid either an excessive or insufficient inflammatory response. These results provide a basis for further study to determine if these aspects of immunity are useful predictors of *Eimeria* resistance in commercial broiler populations, and to establish a phenotypic or genetic trait that can be used as a biomarker for selection.

Reference List

Adami, S., Cavani, A., Rossi, F. and Girolomoni, G. (2014). The role of interleukin-17A in psoriatic disease. *BioDrugs: clinical immunotherapeutics, biopharmaceuticals and gene therapy*, 28(6): 487-497.

Aggarwall, S., Ghilardi, N., Xie, H., De Sauvage, F. J. and Gurney, A. L. (2003). Interleukin-23 promotes a distinct CD4 T cell activation state characterised by the production of interleukin-17 *Journal of Biological Chemistry*, 278(3): 1910-1914.

Aguire, S. A., Perryman, L. E., Davis, W. C. and Mcguire, T. C. (1998). IL-4 protects adult C57BL/6 mice from prolonged Cryptosporidium parvum infection: analysis of CD4+alpha beta+IFN-gamma+ and CD4+alpha beta+IL-4+ lymphocytes in gut-associated lymphoid tissue during resolution of infection. *Journal of Immunology*, 161(4): 1891-1900.

Al-Badri, R. and Barta, J. R. (2012). The kinetics of oocyst shedding and sporulation in two immunologically distinct strains of *Eimeria maxima,* GS and M6. *Parasitology Research*, 111(5): 1947-1952.

Anderson, C. F., Oukka, M., Kuchroo, V. J. and Sacks, D. (2007). $CD4^{+}CD25^{-}Foxp3^{-}$ Th1 cells are the source of IL-10-mediated immune supression in chronic cutaneous leishmaniasis. *Journal of Experimental Medicine*, 204(2): 285-297.

Angata, T., Tabuchi, Y., Nakamura, K. and Nakamura, M. (2007). Siglec-15: an immune system Siglec conserved throughout vertebrate evolution *Glycobiology*, 17(8): 838-846.

Arabkhazaeli, F., Modrisanei, M., Nabian, S., Mansoori, B. and Madani, A. (2013). Evaluation the resistance of *Eimeria* spp. field isolates to anticoccidial drugs using three difference indices. *Iran Journal of Parasitology*, 8(2): 234-241.

Arendt, M. K., Sand, J. M., Marcone, T. M. and Cook, M. E. (2016). Interleukin-10 neutralising antibody for detection of intestinal luminal levels and as a dietary additive in *Eimeria* challanged broiler chicks. *Poultry Science*, 95(2): 430-438.

Avery, S., Rothwell, L., Degen, W. D., Schijns, V. E., Young, J., Kaufman, J. and Kaiser, P. (2004). Characterization of the first nonmammalian T2 cytokine gene cluster: the cluster contains functional single-copy genes for IL-3, IL-4, IL-13, and GM-CSF, a gene for IL-5 that appears to be a pseudogene, and a gene encoding another cytokinelike transcript, KK34. *Journal of Interferon and Cytokine Research*, 24(10): 600-610.

Aylsworth, C. F., Aldhamen, Y. A., Seregin, S. S., Godbehere, S. and Amalfitano, A. (2013). Activation of human natural killer cells by the novel innate immune modulator recombinant *Eimeria* antigen. *Human Immunology*, 74(8): 916-926.

Barin, J. G., Baldeviano, G. C., Talor, M. V., Wu, L., Ong, S., Quader, F., Chen, P., Zheng, D., Caturegli, P., Rose, N. R. and Cihákóvá, D. (2012). Macrophages participate in IL-17-mediated inflammation. *European Journal of Immunology*, 42(3): 726-736.

Befus, A. D., Johnson, N., Leslie, G. A. and Bienenstock, J. (1980). Gut-associated lymphoid tissue in the chicken. I. Morphology, ontogeny, and some functional characteristics of Peyer's patches. *Journal of Immunology*, 125(6): 2626-2632.

Bienenstock, J., Perey, D. Y. E., Gauldie, J. and Underdown, B. J. (1972). Chicken Immunoglobulin Resembling γA. *The Journal of Immunology*, 109(2): 403-406.

Biggs, P. M. (1957). The association of lymphoid tissue with the lymph vessels in the domestic chicken (*Gallus domesticus*). *Acta Anatomica*, 29(1-2): 36-47.

Blake, D. P., Billington, K. J., Copestake, S. L., Oakes, R. D., Quail, M. A., Wan, K. L., Shirley, M. W. and Smith, A. L. (2011). Genetic Mapping Identifies Novel Highly Protective Antigens for an Apicomplexan Parasite. *PLoS One*, 7(2): doi:10.1371/journal.ppat.1001279.

Blake, D. P., Hesketh, P., Archer, A., Shirley, M. W. and Smith, A. L. (2006). *Eimeria maxima*: the influence of host genotype on parasite reproduction as revealed by quantitative real-time PCR. *International Journal for Parasitology*, 36(1): 97-105.

Blake, D. P. and Tomley, F. M. (2014). Securing poultry production from the ever-present *Eimeria* challenge. *Trends in Parasitology*, 30(1): 12-19.

Bockman, D. E. and Cooper, M. D. (1973). Pinocytosis by epithelium associated with lymphoid follicles in the bursa of Fabricius, appendix and Peyer's patches. An electron microscopic study. *American Journal of Anatomy*, 136: 455-478.

Booty, M. G., Barreira-Silva, P., Carpenter, S. M., Nunes-Alves, C., Jacques, M. K., Stowell, B. L., Jayaraman, P., Beamer, G. and Behar, S. M. (2016). IL-21 signalling is essential for optimal host resistance against *Mycobacterium tuberculosis*. *Scientific Reports*: doi: 10.1038/srep36720.

Boyd, A. C., Peroval, M. Y., Hammond, J. A., Prickett, M. D., Young, J. R. and Smith, A. L. (2012). TLR15 is unique to avian and reptilian lineages and recognises a yeast-derived agonist. *Journal of Immunology*, 189(10): 4930-4938.

Brandt, K., Bulfone-Paus, S., Foster, D. C. and Rückert, R. (2003). Interleukin-21 inhibits dendritic cell activation and maturation. *Blood*, 102(12): 4090-4098.

Breed, D. G., Schetters, T. P., Verhoeven, N. A. and Vermeulen, A. N. (1997). Characterisation of phenotype related responsiveness of peripheral blood lymphocytes from *Eimeria tenella* infected chickens. *Parasite Immunology*, 19(12): 563-569.

Brownlie, R., Zhu, J., Allan, B., Mutwiri, G. K., Babiuk, L. A., Potter, A. and Griebel, P. (2009). Chicken TLR21 acts as a functional homologue to mammalian TLR9 in the recognition of CpG oligodeoxynucleotides. *Molecular Immunology*, 46(15): 3163-3170.

Bucy, R. P., Chen, C. L., Cihak, J., Lösch, U. and Cooper, M. D. (1988). Avian T cells expressing gamma delta receptors localise in the splenic sinusoids and the intestinal epithelium. *Journal of Immunology*, 141(7): 2200-2205.

Bumstead, J. M., Bumstead, N., Rothwell, L. and Tomley, F. M. (1995). Comparison of immune responses in inbred lines of chickens to *Eimeria maxima* and *Eimeria tenella*. *Parasitology*, 111(Pt2): 143-151.

Bumstead, N. and Millard, B. J. (1992). Variation in susceptibility of inbred lines of chickens to seven species of *Eimeria*. *Parasitology*, 104(Pt 3): 407-413.

Burkett, P. R., Horste, G. M. and Kuchroo, V. K. (2015). Pouring fuel on the fire: Th17 cells, the environment, and autoimmunity. *The Journal of Clinical Investigation*, 125(6): 2211-2219.

Burns, R. B. (1982). Histology and immunology of Peyer's patches in the domestic fowl (*Gallus domesticus*). *Research in Veterinary Science*, 32(3): 359-367.

Byrnes, S., Emerson, K. and Kogut, M. H. (1993). Dynamics of cytokine production during coccidial infections in chickens: colony-stimulating factors and interferon. *FEMS Immunology and Medical Microbiology*, 6(1): 45-52.

Cai, G., Kastelein, R. A. and Hunter, C. A. (1999). IL-10 enhances NK cell proliferation, cytotoxicity and production of IFN-gamma when combined with IL-18. *European Journal of Immunology*, 29(9): 2658-2665.

Campbell, L. D., Stewart, J. N. and Mead, J. R. (2002). Susceptibility to Cryptosporidium parvum infections in cytokine- and chemokine-receptor knockout mice. *Journal of Parasitology*, 88(5): 1014-1016.

Campos, M. a. S., Almeida, I. C., Takeuchi, O., Akira, S., Valente, E. P., Procópio, D., Travassos, L. R., Smith, J. A., Golenbock, D. T. and Gazzinelli, R. T. (2001). Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. *Journal of Immunology*, 167: 416-423.

Carlander, D. (2002). Avian IgY Antibody: *In vitro* and *in vivo*, University of Uppsala.

Carlander, D., Stålberg, J. and Larsson, A. (1999). Chicken Antibodies. *Upsala Journal of Medical Sciences*, 104: 179-190.

Caruso, R., Fina, D., Peluso, I., Stolfi, C., Fantini, M. C., Gioia, V., Caprioli, F., Blanco, G. V., Paoluzi, O. A., Macdonald, T. T., Pallone, F. and Monteleone, G. (2007). A functional role for interleukin-21 in promoting the synthesis of the T cell chemoattractant MIP-3alpha by gut epithelial cells. *Gastroenterology*, 132(1): 166-175.

Castellano, L. R., Filho, D. C., Argiro, L., Dessein, H., Prata, A., Deissein, A. and Rodrigues, V. (2009). Th1/Th2 immune responses are associated with active cutaneous leishmaniasis and clinical cure is associated with strong interferon-gamma production. *Human Immunology*, 70(6): 383-390.

Chai, J. Y. and Lillehoj, H. S. (1988). Isolation and functional characterisation of chicken intestinal intra-epithelial lymphocytes showing natural killer cell activity against tumour target cells. *Immunology*, 63(1): 111-117.

Challey, J. R. and Burns, W. C. (1959). The invasion of the caecal mucosa by *Eimeria tenella* sporozoites and their transport by macrophages. *Eukaryotic Microbiology*, 6(3): 238-241.

Chapman, H. D. and Shirley, M. W. (2003). The Houghton strain of *Eimeria tenella*: a review of the type of strain selected for genome sequencing. *Avian pathology.* 32(2): 115-127.

Char, D., Sanchez, P., Chen, C. L., Bucy, R. P. and Cooper, M. D. (1990). A third sublineage of avian T cells can be identified with a T cell receptor-3-specific antibody. *Journal of Immunology*, 145(11): 3547-3555.

Chaudhry, A., Samstein, R. M., Treuting, P., Liang, Y., Pils, M. C., Heinrich, J. M., Jack, R. S., Wunderlich, F. T., Brünling, J. C., Müller, W. and Rudensky, A. Y. (2011). Interleukin-10 signalling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. *Immunity*, 34(4): 566-578.

Chen, C. H., Göbel, T. C., Kubota, T. and Cooper, M. D. (1994). T cell development in the chicken. *Poultry Science*, 73(7): 1012-1018.

Chen, C. H., Sowder, J. T., Lahti, J. M., Cihak, J., Lösch, U. and Cooper, M. D. (1989). TCR3: a third T-cell receptor in the chicken. *Proceedings of the National Academy of Sciences of the United States of America*, 86(7): 2351-2355.

Choi, K. D. and Lillehoj, H. S. (2000). Role of chicken IL-2 on gamma delta T-cells and *Eimeria acervulina*-induced changes in intestinal IL-2 mRNA expression and gamma delta T-cells. *Veterinary Immunology and Immunopathology*, 73(3-4): 309-321.

Choi, K. D., Lillehoj, H. S. and Zalenga, D. S. (1999). Changes in local IFN-gamma and TGF-Beta 4 mRNA expression and intraepithelial lymphocytes following *Eimeria* acervulina infection. *Veterinary Immunology and Immunopathology*, 71(3-4): 263-275.

Chow, Y., Wan, K., Blake, D. P., Tomley, F. M. and Nathan, S. (2011). Immunogenic *Eimeria tenella* glycosylphosphatidylinositol-anchored surface antigens (SAGs) induce inflammatory responses in avian macrophages. *PLoS One*, 6(9): e25233.

Chuammitri, P., Ostojić, C., Andreasen, C. B., Redmond, S. B., Lamont, S. J. and Palić, D. (2009). Chicken heterophil extracellular traps (HETs): novel defense mechanism of chicken heterophils. *Veterinary Immunology and Immunopathology*, 129(1-2): 126-131.

Cihak, J., Ziegler-Heitbrock, H. W., Stein, H. and Losch, U. (1991). Effect of perinatal anti-TCR treatment and thymectomy on serum immunoglobulin levels in the chicken. *Zentralblat fur Veterinarmedizin*, 38(1): 28-34.

Clark, E. L., Tomley, F. M. and Blake, D. P. (2017). Are *Eimeria* genetically diverse and does it matter? *Trends in Parasitology*, 33(3): 231-241.

Coban, C., Ishii, K. J., Kawai, T., Hiroaki, H., Sato, S., Uematsu, S., Yamamoto, M., Takeuchi, O., Itagaki, S., Kumar, N., Horii, T. and Akira, S. (2005). Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin *Journal of Experimental Medicine*, 201(1): 19-25.

Cogliani, C., Goossens, H. and Greko, C. (2011). Restricting antimicrobial use in food animals: lessons from Europe. *Microbe*, 6(6): 274-279.

Cooper, M. D., Chen, C. L., Bucy, R. P. and Thompson, C. B. (1991). Avian T cell ontogeny. *Advanced Immunology*, 50: 87-117.

Coquet, J. M., Kyparissoudis, K., Pellicci, D. G., Besra, G., Berzins, S. P., Smyth, M. J. and Godfrey, D. I. (2007). IL-21 is produced by NKT cells and modulates NKT cell activation and cytokine production. *The Journal of Immunology*, 178(5): 2827-2834.

Cornelissen, J. B. W. J., Swinkels, W. J. C., Boersma, W. J. A. and Rebel, J. M. J. (2009). Host response to simultaneous infections with *Eimeria acervulina*, *maxima* and *tenella:* A cumulation of single responses. *Veterinary Parasitology*, 162: 58-66.

Cui, X., Lei, T., Yang, D., Hao, P., Li, B. and Liu, Q. (2012a). *Toxoplasma gondii* immune mapped protein-1 (TgIMP1) is a novel vaccine candidate against toxoplasmosis. *Vaccine*, 30(13): 2282-2287.

Cui, X., Lei, T., Yang, D. Y., Hao, P. and Liu, Q. (2012b). Identification and characterisation of a novel *Neospora caninum* immune mapped protein 1. *Parasitology*, 139(8): 998-1004.

D'andrea, A. M., Rengarajau, M., Valiante, N., Chemini, J., Kubin, M., Aste-Amezaga, M., Chan, S. H., Kobayashi, M., Young, D. A., Nickbarg, R., Chizzonite, R., Wolf, S. F. and Trinchieri, G. (1992). Production of natural killer cell stimulatory factor (NKSF/IL-12) by peripheral blood mononuclear cells. *Journal of Experimental Medicine*, 176(3): 1387-1397.

Dalloul, R. A., Bliss, T. W., Hong, Y. H., Ben-Chouikha, I., Park, D. W., Keeler, C. L. and Lillehoj, H. S. (2007). Unique responses of the avian macrophage to different species of *Eimeria*. *Molecular Immunology*, 44(4): 558-566.

Davis, P. J. and Porter, P. (1979). A mechanism for secretory IgA-mediated inhbition of the cell penetration and intracellular development of *Eimeria tenella*. *Immunology*, 36(3): 471-477.

Davison, F., Magor, K. E. and Kaspers, B. (2008). Structure and evolution of avian immunoglobulins. Avian Immunology. DAVISON, F., KASPERS, B. and SCHAT, K. A. Amsterdam, Elsevier.

Delcacho, E., Gallego, M., Lee, S. H., Lillehoj, H. S., Quilez, J., Lillehoj, E. P. and Sánchez-Acedo, C. (2012). Induction of protective immunity against *Eimeria tenella*, *Eimeria maxima* and *Eimeria acervulina* infections using dendritic cell-derived exosomes. *Infection and Immunity*, 80(5): 1909-1916.

Degen, W. G. J., Van Daal, N., Van Zuilekom, H. I., Burnside, J. and Schijns, V. E. J. C. (2004). Identification and molecular cloning of functional chicken IL-12. *The Journal of Immunology.* 172(7): 4371-4380.

Denyer, M. P., Pinheiro, D. Y., Garden, O. A. and Shepherd, A. J. (2016). Missed, not missing: phylogenomic evidence for the existence of avian FoxP3. *PLoS One*, 11(3): DOI: 10.1371/journal.pone.0150988.

Dhodapkar, B. S., Vegad, J. L. and Kolte, G. N. (1982). Demonstration of the phagocytic activity of chicken basophils in the reversed Arthus reaction using colloidal carbon. *Research in Veterinary Science*, 33(3): 377-379.

Dietert, R. R., Golemboski, K. A., Bloom, S. E. and Qureshi, M. A. (1991). The avian macrophages in cellular immunity. *Avian Cellular Immunity*: 71-95.

Dil, N. and Quereshi, M. A. (2002). Differential expression of inducible nitric oxide synthase is associated with differential Toll-like receptor-4 expression in chicken macroophages from different genetic backgrounds. *Veterinary Immunology and Immunopathology*, 84(3-4): 191-207.

Dimier, I. H., Quéré, P., Naciri, M. and Bout, D. T. (1998). Inhibition of *Eimeria tenella* development in vitro mediated by chicken macrophages and fibroblasts treated with cell supernatants with IFN-gamma activity. *Avian Diseases*, 42(2): 239-247.

Doran, D. J. (1966). The migration of *Eimeria acervulina* sporozoites to the duodenal glands of Lieberkuhn. *Journal of Protozoology*, 13(1): 27-33.

Doran, D. J., Vetterling, J. M. and Augustine, P. C. (1974). *Eimeria tenella*: An in vivo and in vitro comparison of the Wisconsin, Weybridge and Beltsville strains. *Helminthological Society of Washington*, 41(1): 77-80.

Dupont, C. D., Christian, D. A. and Hunter, C. A. (2012). Immune response and immunopathology during *Toxoplasmosis*. *Seminars in Immunology*, 34(6): 793-813.

Ehigiator, H. N., Mcnair, N. and Mead, J. R. (2007). *Cryptosporidium parvum*: the contribution of Th1-inducing pathways to the resolution of infection in mice. *Experimental Parasitology*, 115(2): 107-113.

Elgueta, R., Benson, M. J., Devries, V. C., Wasiuk, A., Guo, Y. and Noelle, R. J. (2009). Molceular mechanism and function of CD40/CD40L engagement in the immune system. *Immunological Reviews*, 229(152-172).

Elsaesser, H., Sauer, K. and Brooks, D. G. (2009). IL-21 is requred to control chronic viral infection. *Science*, 324(5934): 1569-1572.

Ely, K. H., Kasper, L. H. and Khan, I. A. (1999). Augmentation of the CD8+ T cell response by IFN-gamma in IL-12- deficient mice during Toxoplasma gondii infection. *Journal of Immunology*, 162(9): 5449-5454.

Ettinger, R., Sims, G. P., Fairhurst, A. M., Robbins, R., Da Silva, Y. S., Spolski, R., Leonard, W. J. and Lipsky, P. E. (2005). IL-21 induces differentiation of human naive

and memory B cells into antibody-secreting plasma cells. *Journal of immunology*, 175(12): 7867-7879.

Evans, E. W., Beach, G. G., Wunderlich, J. and Harmon, B. G. (1994). Isolation of antimicrobial peptides from avian heterophils. *Journal of Leukocyte Biology*, 56(5): 661-665.

Ferdous, F., Maurice, D. and Scott, T. (2008). Broiler chick thrombocyte response to lipopolysaccharide. *Poultry Science*, 87(1): 61-63.

Fetterer, R. H., Miska, K., Jenkins, M. C. and Barfield, R. C. (2004). A conserved 19-kDa *Eimeria tenella* antigen is a profilin-like protein. *Journal of Parasitology*, 90(6): 1321-1328.

Fina, D., Sarra, M., Fantini, M. C., Rizzo, A., Caruso, R., Caprioli, F., Stolfi, C., Cardolini, I., Dottori, M., Boirivant, M. and Pallone, F. (2008). Regulation of gut inflammation and Th17 cell response by interleukin-21. *Gastroenterology*, 134(4): 1038-1048.

Fröhlich, A., Kisielow, J., Schmitz, I., Freigang, S., Shamshiev, A. T., Weber, J., Marsland, B. J., Oxenius, A. and Kopf, M. (2009). IL-21R on T cells is critical for sustained functionality and control of chronic viral infection. *Science*, 324(5934): 1576-1580.

Fumgalli, S., Torri, A., Papagna, A., Citterio, S., Mainoldi, F. and Foti, M. (2016). IL-22 is rapidly induced by Pathogen Recognition Receptors Stimulation in Bone-Marrow-derived Dendritic Cells in the Absence of IL-23. *Scientific Reports*, 22(6): doi: 10.1038/srep33900.

Fuss, I. J., Heller, F., Boirivant, M., Leon, F., Yoshida, M., Fichtner-Feigl, S., Yang, Z., Exley, M., Kitani, A., Blumberg, R. S., Mannon, P. and Strober, W. (2004). Nonclassical CD1d-restricted NK T cells that produce IL-13 chatcerise an atypical Th2 response in ulcerative colitis. *Journal of Clinical Investigation*, 113(10): 1490-1197.

Galli, S. J., Nakae, S. and Tsai, M. (2005). Mast cells in the development of adaptive immune responses. *Nature Immunology*, 6(2): 135-142.

Garceau, V., Smith, J., Paton, I. R., Davey, M., Fares, M. A., Sester, D. P., Burt, D. W. and Hume, D. A. (2010). Pivotal advance: Avian colony stimulating factor 1 (CSF-1), interleukin-34 (IL-34) and CSF-1 receptor genes and gene products. *Journal of Leukocyte Biology*, 87(5): 753-764.

Garcia-Morales, C., Rothwell, L., Moffat, L., Garcaeu, V., Balic, A., Sang, H. M., Kaiser, P. and Hume, D. A. (2014). Production and characterisation of a monoclonal antibody that recognises the chicken CSF1 receptor and confirms that expression is restricted to macrophage-lineage cells. *Developmental and Comparative Immunology*, 42(2): 278-285.

Gazzinelli, R. T., Wysocka, M., Hieny, S., Shcarton-Kersten, T., Cheever, A., Kühn, R., Müller, W., Trinchieri, G. and Sher, A. (1996). In the absence of endogenous IL-10, mice acutely infected with *Toxoplasma gondii* succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. *Journal of Immunology*, 157(2): 798-805.

Ghilardi, N. and Ouyang, W. (2007). Targeting the development and effector functions of Th17 cells. *Seminars in Immunology*, 19(6): 383-393.

Ghosh, D. and Stumhofer, J. S. (2013). Do you see what I see: Recognition of protozoan parasites by Toll-like receptors. *Current Immunology Reviews*, 9(3): 129-140.

Göbel, T. W., Chen, C. L., Shrimpf, J., Grossi, C. E., Bernot, A., Bucy, R. P., Auffray, C. and Cooper, M. D. (1994). Characterisation of avian natural killer cells and their intracellular CD3 protein complex. *European Journal of Immunology*, 24(7): 1685-1691.

Göbel, T. W., Kaspers, B. and Stangassinger, M. (2001). NK and T cells constitute two major, functionally distinct intestinal epithelial lymphocyte subsets in the chicken. *International Immunology*, 13(6): 757-762.

Godfrey, D. I., Stankovic, S. and Baxter, A. G. (2010). Raising the NKT cell family. *Nature Immunology*, 11(3): 197-206.

Goswami, R., Jabeen, R., Yagi, R., Pham, D., Zhu, J., Goenka, S. and Kaplan, M. H. (2012). STAT6-dependent regulation of Th9 development. *Journal of Immunology*, 188(3): 968-975.

Goto, M., Murakawa, M., Kadoshima-Yamaoka, K., Tanaka, Y., Nagahira, K., Fukuda, Y. and Nishimura, T. (2009). Murine NKT cells produce Th17 cytokine interleukin-22. *254*, 2(81-84).

Gowen, B. B., Judge, J. W., Wong, M. H., Jung, K. H., Aylsworth, C. F., Melby, P. C., Rosenber, B. and Morrey, J. D. (2008). Immunoprophylaxis of Punta Toro virus (Phlebovirus, Bunyaviridae) infection in hamsters with recombinant *Eimeria* profilin-like antigen. *International Immunopharmacology*, 8(8): 1089-1094.

Gu, Y., Yang, J., Outang, X., Liu, W., Li, H., Yang, J., Bromberg, J., Chen, S., Mayer, L., Unkeless, J. C. and Xiong, H. (2008). Interleukin 10 suppresses Th17 cytokines secreted by macrophages and T cells. *European Journal of Immunology*, 38(7): 1807-1813.

Guedes, P. M. D. M., Gutierrez, F. R., Maia, F. L., Milanezi, C. M., Silva, G. K., Pavanelli, W. R. and Silva, J. S. (2010). IL-17 produced during *Trypanasoma cruzi* infection plays a central role in regulating parasite-induced myocarditis. *PLos Neglected Tropical Diseases*, 4(2): DOI: 10.1371/journal.pntd.0000604.

Gum, J. R., Hicks, J. W., Toribara, N. W., Siddiki, B. and Kim, Y. S. (1994). Molecular cloning of human intestinal mucin (MUC2) cDNA. Identification of the amino terminus and overall sequence similarity to prepro-von Willebrand factor. *Journal of Biological Chemistry*, 269(4): 2440-2446.

Guo, P., Thomas, J. D., Bruce, M. P., Hinton, T. M., Bean, A. G. and Lowenthal, J. W. (2013). The chicken TH1 response: potential therapeutic applications of ChIFN-γ. *Developmental and Comparative Immunology*, 41(3): 389-396.

Hála, K., Schauenstein, K., Neu, N., Krömer, G., Wolf, H., Böck, G. and Wick, G. (1986). A monoclonal antibody reacting with a membrane determinant expressed on activated chicken T lymphocytes. *European Journal of Immunology*, 16(11): 1331-1336.

Halper, J., Burt, D. W. and Romanov, M. N. (2004). On reassessment of the chicken TGFB4 gene as TGFB1. *Growth Factors*, 22(2): 121-122.

Harrington, L. E., Hatton, R. D., Mangan, P. R., Turner, H., Murphy, T. L., Murphy, K. M. and Weaver, C. T. (2005). Interleukin 17-producing $CD4^+$ T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nature Immunology*, 6(11): 1123-1132.

He, H., Crippen, T. L., Farnell, M. B. and Kogut, M. H. (2003). Identification of CpG oligodeoxynucleotide motifs that stimulate nitric oxide and cytokine production in avian macrophage and peripheral blood mononuclear cells. *Developmental and Comparative Immunology*, 27(6-7): 621-627.

He, H., Genovese, K. H. and Kogut, M. H. (2011). Modulation of chicken macrophage effector function by T(H)1/T(H)2 cytokines. *Cytokine*, 53(3): 363-369.

Hedge, S. N., Wagenaar, F. and Janse, E. M. (1999). Further characterisation of M cells in gut-associated lymphoid tissues of the chicken. *Poultry Science*, 78(965-972).

Higuchi, M., Matsuo, A., Shingai, M., Shida, K., Ishii, A., Funami, K., Suzuki, Y., Oshiumi, H., Matsumoto, M. and Seya, T. (2008). Combinational recognition of bacterial lipoproteins and peptidoglycan by chicken Toll-like receptor 2 subfamily. *Developmental and Comparative Immunology*, 32(2): 147-155.

Hirano, M., Das, S., Guo, P. and Cooper, M. D. (2011). The evolution of adaptive immunity in vertebrates. *Adnacnces in Immunology*, 109: 125-157.

Hoan, T. D., Thao, D. T., Gadahi, J. A., Song, X., Xu, L., Yan, R. and Li, X. (2014). Analysis of humoral immune response and cytokines in chickens vaccinated with *Eimeria brunetti* apical membrane antigen 1 (EbAMA1) DNA vaccine. *Experimental Parasitology*, 144: 65-72.

Hoan, T. D., Zhang, Z., Huang, J., Yan, R., Song, X., Xu, L. and Li, X. (2016). Identification and immunogenicity of microneme protein 2 (EbMIC2) of *Eimeria brunetti*. *Experimental Parasitology*, 162: 7-17.

Hoggenmueller, L., Wakenell, P. S. and Schat, K. A. (1993). Preparation and characterisation of chicken intraepithelial leukocytes. *Avian Pathology*, 22(3): 509-523.

Holmskov, U. and Jensenius, J. C. (1993). Structure and function of collectins: humoral C-type lectins with collagenous regions. *Behring Institute Mitteilungen*, 93(224-235).

Hong, Y. H., Lillehoj, H. S., Lee, S. H., Dalloul, R. A. and Lillehoj, E. P. (2006a). Analysis of chicken cytokine and chemokine gene expression following *Eimeria acervulina* and *Eimeria tenella* infections. *Veterinary Immunology and Immunopathology*, 114(3-4): 209-223.

Hong, Y. H., Lillehoj, H. S., Lillehoj, E. P. and Lee, S. H. (2006b). Changes in immune-related gene expression and intestinal lymphocyte subpopulations following *Eimeria maxima* infection of chickens. *Veterinary Immunology and Immunopathology*, 114(3-4): 259-272.

Hot, A. and Miossec, P. (2011). Effects of interleukin (IL)-17A and IL-17F in human rheumatoid arthritis synoviocytes. *Annals of the Rheumatic Diseases*, 70(5): 727-732.

Houssaint, E., Diez, E. and Pink, J. R. L. (1987). Ontogen and tissue distribution of the chicken Bu-1a antigen. *Immunology*, 62(3): 463-470.

Hu, T., Wu, Z., Vervelde, L., Rothwell, L., Hume, D. A. and Kaiser, P. (2016). Functional annotation of the T cell immunoglobulin mucin (TIM) family in birds. *Immunology*.

Hu, Y., Shen, F., Crellin, N. K. and Ouyang, W. (2011). The IL-17 pathway as a major therapeutic target in autoimmune diseases. *Annals of the New York Academy of Science*, 1217: 60-76.

Huang, J., Zhang, Z., Li, M., Song, X., Yan, R., Xu, L. and Li, X. (2015). Immune protection of microneme 7 (EmMIC7) against *Eimeria maxima* challenge in chickens. *Avian Pathology*, 44(5): 392-400.

Hunter, C., Candolfi, E., Subauste, C., Van Cleave, V. and Remington, J. (1995). Studies on the role of interleukin-12 in acute murine toxoplasmosis. *Immunology*, 84: 16-20.

Ingulli, E., Mondino, A., Khoruts, A. and Jenkins, M. K. (1997). *In vivo* detection of dendritic cell antigen presentation to $CD4^+$ T cells. *Journal of Experimental Medicine*, 185(12): 2133-2141.

Iqbal, M., Philbin, V., Withanage, G. S. K., Wigley, P., Beal, R. K., Goodchild, M., Barrow, P., Mcconnell, I., Maskell, D. J., Young, J., Bumstead, N., Boyd, Y. and Smith, A. L. (2005). Identification and functional characterisation of chicken Toll-like receptor 5

reveals a fundamental role in the biology of infection with *Salmonella enterica* serovar Typhimurium. *Infection and Immunity*, 73(4): 2344-2350.

Ishigame, H., Kakuta, S., Nagai, T., Kadoki, M., Nambu, A., Komiyama, Y., Fujikado, N., Tanahashi, Y., Akitschu, A., Kotaki, H., Sudo, K., Nakae, S., Sasakawa, C. and Iwakura, Y. (2009). Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses. *Immunity*, 30(1): 108-119.

Ivanov, I. I., Mckenzie, B., Zhou, L., Tadokoro, C. E., Lepelley, A., Lafaille, J. J., Cua, D. J. and Littman, D. R. (2006). The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17^{+} T helper cells. *Cell*, 126(6): 1121-1133.

Ivanov, I. I., Zhou, L. and Littman, D. R. (2007). Transcriptional regulation of Th17 cell differentiation. *Seminars in Immunology*, 19(6): 409-417.

Jakowlew, S. B., Mathias, A. and Lillehoj, H. S. (1997). Transforming growth factor-beta isoforms in the developing chicken intestin and spleen: increase in transforming growth factor-beta 4 with coccidia infection. *Veterinary Immunology and Immunopathology*, 55(4): 321-339.

Jankovic, D., Kullberg, M. C., Feng, C. G., Goldszmid, R. S., Collazo, C. M., Wilson, M., Wynn, T. A., Kamanaka, M., Flavell, R. A. and Sher, A. (2007). Conventional T-bet^{+}Foxp3^{-} Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection. *Journal of Experimental Medicine*, 204(2): 273-283.

Jansen, C. A., Van De Haar, P. M., Van Haarlem, D., Van Kooten, P., De Wit, S., Van Eden, W., Viertlböck, B. C., Göbel, T. C. and Vervelde, L. (2010). Identification of new populations of chicken natural killer (NK) cells. *Developmental and Comparative Immunology*, 34(7): 759-767.

Janzarik, H. and Morgenstern, E. (1979). The nucleated thrombocytoid cells. I. Electron microscopic studies on chicken blood cells. *Thrombosis and Haemostasis*, 41(3): 608-621.

Jenkins, M. C., Dubey, J. P., Miska, K. and Fetterer, R. (2017). Differences in decundity of *Eimeria maxima* strains exhibiting different levels of pathogenicity in its avian host. *Veterinary Parasitology*. 236: 1-6.

Jenkins, M. C., Fetterer, R., Miska, K., Tuo, W., Kwok, O. and Dubey, J. P. (2015). Characterisation of the *Eimeria maxima* sporozoite surface protein IMP1. *Veterinary Parasitology*, 211(3-4): doi: 10.1016/j.vetpar.2015.1005.1009.

Jeurissen, S. H., Claassen, E. and Janse, E. M. (1992). Histological and functional differentiation of non-lymphoid cells in the chicken spleen. *Immunology*, 77(1): 75-80.

Jeurissen, S. H., Janse, E. M., Koch, G. and Deboer, G. F. (1989). Postnatal development of mucosa-associated lymphoid tissues in chickens. *Cell Tissue Research*, 258(1): 119-124.

Jeurissen, S. H., Janse, E. M., Vermeulen, A. N. and Vervelde, L. (1996). *Eimeria tenella* infections in chickens: aspects of host parasite interaction. *Veterinary Immunology and Immunopathology*, 54(1-4): 231-238.

Jeurissen, S. H., Wagenaar, F. and Janse, E. M. (1999). Further characterisation of M cells in gut-associated lymphoid tissues of the chicken. *Poultry Science*, 78(7): 965-972.

Jiang, L., Lin, J., Han, H., Dong, H., Zhao, Q., Zhu, S. and Huang, B. (2012). Identification and characterisation of *Eimeria tenella* apical membrane antigen 1 (AMA1). *PLoS One*, 7(7): doi: 10.1371/journal.pone.0041115.

Jiang, Z., Applegate, T. J. and Lossie, A. C. (2013). Cloning, annotation and developmental expression of the chicken intestinal MUC2 gene. *PLoS One*, 8(1): DOI: 10.1371/journal.pone.0053781.

Johnson, J. and Reid, W. M. (1970). Anticoccidial drugs: lesion scoring techniques in battery and floor-pen experiments with chickens. *Experimental Parasitology*, 28(1): 30-36.

Jovanovic, D. V., Bibattista, J. A., Martel-Pelletier, J., Jolicoeur, F. C., He, Y., Zhang, M., Mineau, F. and Pelletier, J. P. (1998). IL-17 stimulates the production and expression of proinflammatory cytokines, IL-1beta and TNF-alpha, by human macrophages. *Journal of Immunology*, 160(7): 3513-3535-3521.

Joyner, L. P. and Norton, C. C. (1969). A comparison of two laboratory strains of *Eimeria tenella*. *Parasitology*, 59: 907-913.

Kaiser, P., Poh, T. Y., Rothwell, L., Avery, S., Balu, S., Pathania, U. S., Hughes, S., Goodchild, M., Morrell, S., Watson, M., Bumstead, N., Kaufman, J. and Young, J. R. (2005). A genomic analysis of chicken cytokines and chemokines. *Journal of the International Society for Interferon and Cytokine Research*, 25(8): 467-484.

Kaliner, M. (1987). Mast cell mediators and asthma. *Chest*, 91(6): 171-176.

Karpala, A. J., Stewart, C., Mckay, J., Lowenthal, J. W. and Bean, A. G. (2011). Characterisation of chicken Mda5 activity: regulation of IFN-beta in the absense of RIG-I functionality. *Journal of Immunology*, 186(9): 5397-5405.

Kelly, M. N., Kolls, J. K., Happel, K., Schwartzman, J. D., Schwarzenberger, P., Combe, C., Moretto, M. and Khan, I. A. (2005). Interleukin-17/interleukin-17 receptor-mediated signalling is important for generation of an optimal polymorphonuclear response against *Toxoplasma gondii* infection. *Infection and Immunity*, 73(1): 617-621.

Kim, S., Faris, L., Cox, C. M., Sumners, L. H., Jenkins, M. C., Fetterer, R., Miska, K. and Dalloul, R. A. (2012). Molecular characterisation and immunological roles of avian IL-22 and its soluble receptor IL-22 binding protein. *Cytokine*, 60(3): 815-827.

Kim, W., Jeong, J., Park, A. R., Yim, D., Kim, Y. H., Kim, K. D., Chang, H. H., Lillehoj, H. S., Lee, B. H. and Min, W. (2012). Chicken IL-17F: identification and comparative expression analysis in *Eimeria*-infected chickens. *Developmental and Comparative Immunology*, 38(3): 401-409.

Kim, W. H., Jeong, J., Park, A. R., Yim, D., Kim, S., Chang, H. H., Yang, S. H., Kim, D. H., Lillehoj, H. S. and Min, W. (2014). Downregulation of chicken interleukin-17 receptor A during *Eimeria* infection. *Infection and Immunity*, 82(9): 3845-3854.

Kinugasa, T., Sakaguchi, T., Gu, X. and Reinecker, H. (2000). Claudins regulate the intestinal barrier in response to immune mediators. *Gastroenterology*, 118(6): 1001-1011.

Klasing, K. C. (1998). Avian macrophages: regulators of local and systemic immune responses. *Poultry Science*, 77(7): 983-989.

Kogut, M. H. (2002). Dynamics of a protective avian inflammatory response: the role of an IL-8-like cytokine in the recruitment of heterophils to the site of organ invasion by Salmonella enteritidis. *Comparative Immunology, Microbiology and Infectious Disease*, 25(3): 159-172.

Kogut, M. H., Iqbal, M., He, H., Philbin, V., Kaiser, P. and Smith, A. (2005a). Expression and function of Toll-like receptors in chicken heterophils. *Developmental and Comparative Immunology*, 29(791-807).

Kogut, M. H., Rothwell, L. and Kaiser, P. (2005b). IFN-gamma priming of chicken heterophils upregulates the expression of proinflammatory and Th1 cytokine mRNA following receptor-mediated phagocytosis of *Salmonella enterica* serovar enteritidis. *Journal of the International Society for Interferon and Cytokine Research*, 25(2): 73-81.

Kogut, M. H., Tellez, G. I., Mcgurder, E. D., Hargis, B. M., Williams, J. D., Corrier, D. E. and Deloach, J. R. (1994). Heterophils are decisive components of the early responses of chickens to *Salmonella enteritidis* infections. *Microbial Pathogenesis*, 16(2): 141-151.

Korn, T., Bettelli, E., Oukka, M. and Kuchroo, V. K. (2009). IL-17 and Th17 cells. *Annual Review of Immunology*, 27: 485-517.

Kouro, T. and Takatsu (2009). IL-5- and eosinophil-mediated inflammation: from discovery to therapy. *International Immunology*, 21(12): 1303-1309.

Kowalczyk, K., Daiss, J., Halpern, J. and Roth, T. (1985). Quantitation of maternal-fetal IgG transport in the chicken. *Immunology*, 54(4): 755-762.

Krause, P., Morris, V., Greenbaum, J. A., Park, Y., Biorheden, U., Mikulski, Z., Muffley, T., Shui, J. W., Kim, G., Cheroute, H., Liu, Y. C., Peters, B., Kronenberg, M. and Murai, M. (2015). IL-10-producing intestinal macrophages prevent excessive antibacterial innate immunity by limiting IL-23 synthesis. *Nature Communications*, 6: doi: 10.1038/ncomms8055.

Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K. and Muller, W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*, 75(2): 263-274.

Kwa, S. F., Beverley, P. and Smith, A. (2006). Peyer's patches are required fro the induction of rapid Th1 responses in the gut and mesenteric lymph nodes during and enteric infection. *Journal of immunology*, 176(12): 7533-7541.

Laidlaw, B. J., Cui, W., Amezquita, R. A., Gray, S. M., Guan, T., Lu, Y., Kobayashi, Y., Flavell, R. A., Kleinstein, S. H., Craft, J. and Kaech, S. M. (2015). Production of IL-10 by CD4+ regulatory T cells during the resolution of infections promotes maturation of memory CD8+ T cells. *Nature Immunology*, 16(8): 871-879.

Lam, K. M. (2002). The macrophage inflammatory protein-1beta in the supernatants of *Mycoplasma gallisepticum*-infected chicken leukocytes attracts the migrations of chicken heterophils and lymphocytes. *Developmental and Comparative Immunology*, 26(1): 85-93.

Laurent, F., Mancassola, R., Lacroix, S., Menezes, R. and Naciri, M. (2001). Analysis of chicken mucosal immune response to *Eimeria tenella* and *Eimeria maxima* infection by quantitative reverse transcription-PCR. *Infection and Immunity*, 69(4): 2527-2534.

Lawn, A. M. and Rose, M. E. (1982). Mucosal transport of *Eimeria tenella* in the caecum of the chicken. *Journal of Parasitology*, 68(6): 1117-1123.

Lebacq-Verheyden, A. M., Vaerman, J. P. and Heremans, J. F. (1972). A possible homologue of mammalian IgA in chicken serum and secretions. *Immunology*, 22(1): 165-175.

Lee, W. K., Lillehoj, H. S., Jang, S., Lee, S. H., Bautista, D. A., Ritter, G. D., Lillehoj, E. P. and Siragusa, G. R. (2013). Comparison of live *Eimeria* vaccination with in-feed salinomycin on growth and immune status in broiler chickens. *Research in Veterinary Science*, 95(110-114).

Lehmann, J., Enssle, K. H., Lehmann, I., Emmendörfer, A. and Lohmann-Matthes, M. L. (2000). The capacity to produce IFN-gamma rather than the presence of interleukin-4 determines the resistance and the degree of susceptibility to *Leishmania donovani*

infection in mice. *Journal of International Society for Interferon and Cytokine Research*, 2-(1): 63-77.

Li, S., Wang, W., Fu, S., Wang, J., Liu, H., Xie, S., Liu, B., Li, Y., Lv, Q., Li, Z., Xue, W., Huang, B., Chen, W. and Liu, J. (2013). IL-21 modulates release of proinflammatory cytokines in LPS-stimulated macrophages through distinct signalling pathways. *Mediators of Inflammation*: doi: 10.1155/2013/548073.

Li, S., Zhang, M. Z., Yan, L., Lillehoj, H. S., Pace, L. W. and Zhang, S. (2009). Induction of CXC chemokine messenger-RNA expression in chicken oviduct epithelial cells by *Salmonella enterica* serovar enteriditis via the type three secretion system-1. *Avian Diseases*, 53(3): 396-404.

Li, Y., Ingmer, H., Madsen, M. and Bang, D. D. (2008). Cytokine resonses in primary chicken embryo intestinal cells infected with *Campylobacter jejuni* strains of human and chicken origin and the expression of bacterial virulence-associated genes. *BMC Microbiology*, 8(107): doi: 10.1186/1471-2180-1188-1107.

Li, Z. Y., Chen, J., Petersen, E., Zhou, D. H., Huang, S. Y., Song, H. Q. and Zhu, X. Q. (2014). Synergy of mIL-21 and mIL-15 in enhancing DNA vaccine efficacy against acute and chronic *Toxoplasma gondii* infection in mice. *Vaccine*, 32(25): 3058-3065.

Lillehoj, H. S. (1987). Effects of immunosuppression on avian coccidiosis: cyclosporin A but not hormonal bursectomy abrogates host protective immunity. *Infection and Immunity*, 55(7): 1616-1621.

Lillehoj, H. S. (1989). Intestinal intraepithelial and splenic natural killer cell responses to *Eimerian* infections in inbred chickens. *Infection and Immunity*, 57(7): 1879-1884.

Lillehoj, H. S. and Li, G. (2004). Nitric oxide production by macrophages stimulated with coccidia sporozoites, lipopolysaccharide, or interferon-gamma, and its dynamic changes in SC and TK strains of chickens infected with *Eimeria tenella*. *Avian Diseases*, 48(2): 244-253.

Liniger, M., Summerfield, A., Zimmer, G., Mccullough, K. C. and Ruggli, N. (2012). Chicken cells sense influenza A virus infection through MDA5 and CARDIF signalling involving LGP2. *Journal of Virology*, 86(2): 705-717.

Liu, H., Zhang, M., Han, H., Yuan, J. and Li, Z. (2010). Comparison of the expression of cytokine genes in the bursal tissues of the chickens following challenge with infectious bursal disease viruses of varying virulence. *Virology Journal*, 7(1): DOI: 10.1186/1743-1422X-1187-1364.

Lizundia, R., Sauter, K. S., Taylor, G. and Werling, D. (2008). Host species-specific usage of the TLR4-LPS receptor complex. *Innate Immunity*, 14(4): 223-231.

Ljunggren, H. G. and Karre, K. (1990). In search of the 'missing self': MHC molecules and NK cell recognition. *Immunology Today*, 11(7): 237-244.

Long, P. L. (1959). A study of *Eimeria maxima,* Tyzzer 1929, a coccidium of the fowl (*Gallus gallus*). *Annals of Tropical Medicine and Parasitology.* 53: 325-333.

Long, P. L. (1968a). The effect of breed of chickens on resistance to *Eimeria* infections. *British Poultry Science*, 9(1): 71-78.

Long, P. L. (1968b). The pathogenic effects of *Eimeria praecox* and *E. acervulina* in the chicken. *Parasitology*, 58(3): 691-700.

Long, P. L. (1974). Experimental infection of chickens with two species of *Eimeria* isolated fromt he Malaysian jungle fowl. *Parasitology*, 69(3): 337-347.

Long, P. L. and Joyner, L. P. (1984). Problems in the identification of species of *Eimeria*. *Journal of Protozoology*, 31(4): 535-541.

Long, P. L. and Millard, B. J. (1976). Studies on site finding and site specificity of *Eimeria praecox*, *Eimeria maxima* and *Eimeria acervulina* in chickens. *Parasitology*, 73(3): 327-336.

Lowenthal, J. W., York, J., O'neil, T. E., Rhodes, S., Prowse, S. J., Strom, D. G. and Digby, M. R. (1997). *In vivo* effects of chicken interferon-gamma during infection with *Eimeria*. *Journal of the International Society for Interferon and Cytokine Research*, 17(9): 551-558.

Luhtala, M., Lassila, O., Toivanen, P. and Vainio, O. (1992). A novel peripheral CD4+CD8+ T cell population: inheritance of CD8alpha expression on CD4+ T cells.

Lynagh, G. R., Bailey, M. and Kaiser, P. (2000). Interleukin-6 is produced during both murine and avian *Eimeria* infections. *Veterinary Immunology and Immunopathology*, 76(1-2): 89-102.

Ma, C. S., Deenick, E. K., Batten, M. and Tangye, S. G. (2012). The origins, function and regulation of T follicular helper cells. *Journal of Experimental Medicine*, 209(7): 1241-1253.

Maeda, M., Yanagawa, Y., Iwabuchi, K., Minami, K., Nakamaru, Y., Takagi, D., Fukuda, S. and Onoé, K. (2007). IL-21 enhances dendritic cell ability to induce interferon-gamma production by natural killer T cells. *Immunobiology*, 212(7): 537-547.

Mantovani, A. and Marchesi, F. (2014). IL-10 and macrophages orchestrate gut homeostasis. *Immunity*, 40(5): 637-639.

Martin, B., Hirota, K., Cua, D. J., Stockinger, B. and Veldhoen, M. (2009). Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. *Immunity*, 31(2): 321-330.

Martínez-López, M., Iborra, S., Conde-Garrosa, R. and Sancho, D. (2015). Batf3-dependent CD103+ dendritic cells are major producers of IL-12 that drive local Th1 immunity against *Leishmania major* infection in mice. *European Journal of Immunology*, 45(1): 119-129.

Marugan-Hernandez, V., Long, E., Blake, D. P., Crouch, C. and Tomley, F. M. (2017). *Eimeria tenella* protein trafficking: differential regulation of secretion versus surface tethering during the life cycle. *Scientific Reports*, 7(1): doi: 10.1038/s41598-41017-04049-41591.

Mast, J., Goddeeris, B. M., Peeters, K., Vandesande, F. and Berghman, L. R. (1998). Characterisation of chicken monocytes, macrophages and interdigitating cells by the monoclonal antibody KUL01. *Veterinary Immunology and Immunopathology*, 61(2-4): 343-357.

Mathur, R., Oh, H., Zhang, D., Park, S. G., Seo, J., Koblansky, A., Hayden, M. S. and Ghosh, S. (2012). A mouse model of *Salmonella typhi* infection. *Cell*, 151(3): 590-602.

Maxwell, M. H. (1987). The avian eosinophil - a review. *Poultry Science*, 43(3): 190-207.

Mcdougald, L. R. (1998). Intestinal protozoa important to poultry. *Poultry Science*, 77(8): 1156-1158.

Merkle, H., Cihak, J. and Losch, U. (1992). The cytotoxic T lymphocyte response in reticuloendotheliosis virus-infected chickens is mediated by alpha beta and not by gamma delta T cells. *Immunobiology*. 186(3-4):292-303.

Merlino, P. G. and Marsh, J. A. (2002). The enhancement of avian NK cell cytotoxicity by thymulin is not mediated by the regulation of IFN-gamma production. *Developmental and Comparative Immunology*, 26(1): 103-110.

Millard, B. J., Bradley, J. W. A. and Long, P. L. (1971). The schizongony of *Eimeria maxima* in the chicken. *Zeitschrift für Parasitekunde.* 38(1): 77-81.

Mills, C. D., Kincaid, K., Alt, J. M., Heilman, M. J. and Hill, A. M. (2000). M-1/m-2 macrophages and the Th1/Th2 paradigm. *The Journal of Immunology*, 164(12): 6166-6173.

Mills, K. H. G. (2008). Induction, function and regulation of IL-17-producing T cells. *European Journal of Immunology*, 38(10): 2636-2649.

Min, W., Kim, W. H., Lillehoj, E. P. and Lillehoj, H. S. (2013). Recent progress in host immunity to avian coccidiosis: IL-17 family cytokines as sentinels of the intestinal mucosa. *Developmental and Comparative Immunology*, 41(3): 418-428.

Minns, L. A., Menard, L. C., Foureau, D. M., Darche, S., Ronet, C., Mielcarz, D. W., Buzoni-Gatel, D. and Kasper, L. H. (2006). TLR9 is required for the gut-associated lymphoid tissue response following oral infection with *Toxoplasma gondii*. *Journal of Immunology*, 176: 7589-7597.

Miska, K. B., Kim, S., Fetterer, R., Dalloul, R. A. and Jenkins, M. C. (2013). Macrophage migration inhibitory factor (MIF) of the protozoan parasite *Eimeria* influences the components of the immune system of its host, the chicken. *Parasitology Research*, 112(5): 1935-1944.

Mittal, A., Murugaiyan, G., Beynon, V., Hu, D. and Weiner, H. L. (2012). IL-27 induction of IL-21 from human CD8 T cells induces granzyme B in an autocrine manner. *Immunology and Cell Biology*, 90(8): 831-835.

Mocellin, S., Panelli, M., Wang, E., Rossi, C. R., Pilati, P., Nitti, D., Lise, M. and Marincola, F. M. (2004). IL-10 stimulatory effects on human NK cells explored by gene profile analysis. *Genes and Immunity*, 5(8): 621-630.

Monteleone, G., Caruso, R., Fina, D., Peluso, I., Gioia, V., Stolfi, C., Fantini, M. C., Caprioli, F., Tersigni, R., Alessandroni, L., Macdonald, T. T. and Pallone, F. (2006). Control of matrix metalloproteinase production in human intestinal fibroblasts by interleukin 21. *Gut*, 55(12): 1774-1780.

Monteleone, G., Monteleone, I., Fina, D., Vavassori, P., Blanco, G. V., Caruso, R., Tersigni, R., Alessandroni, L., Biancone, L., Naccari, G. C., Macdonald, T. T. and Pallone, F. (2005). Interleukin-21 enhances T helper cell type I signalling and interferon-gamma production in Crohn's disease. *Gastroenterology*, 128(3): 687-694.

Mosmann, T. R. and Coffman, R. L. (1989). Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annual Review of Immunology*, 7: 145-173.

Mou, Z., Jia, P., Kuriakose, S., Khadem, F. and Uzonna, J. E. (2010). Interleukin-17-meidated control of parasitemia in experimental *Trypanosoma* congolense infection in mice. *Infection and Immunity*, 78(12): 5271-5279.

Mullen, A. C., High, F. A., Hutchins, A. S., Lee, H. W., Villarino, A. V., Livingston, D. M., Kung, A. L., Cereb, N., Yao, T. P., Yang, S. Y. and Reiner, S. L. (2001). Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science*, 292(5523): 1907-1910.

Murphy, M. L., Wille, U., Villegas, E. N., Hunter, C. A. and Farrell, J. P. (2001). IL-10 mediates susceptibility to *Leismania donovani* infection. *European Journal of Immunology*, 31(10): 2848-2856.

Nagumo, H., Abe, J., Kano, H., Taki, S., Yamazaki, T., Kobayashi, N., Koike, K., Sugane, K., Saito, H. and Agematsu, K. (2009). Distinct response in maintenance of human naive and memory B cells via IL-21 receptor and TCL1/Akt pathways. *Cellular Immunology*, 256(1-2): 56-63.

Nakao, Y., Funami, K., Kikiawa, S., Taniguchi, M., Nishiguchi, M., Fukumori, Y., Seya, T. and Matsumoto, M. (2005). Surface-expressed TLR6 participates in the recognition of diacylated lipopeptide and peptidoglycan in human cells. *Journal of Immunology*, 174(3): 1566-1573.

Neulen, M. L., Viertlboeck, B. C., Straub, C. and Göbel, T. C. (2015). Identification of novel chicken CD4+ CD3+ blood population with NK cell like features. *Developmental and Comparative Immunology*, 49(1): 72-78.

Nishiguchi, M., Matsumoto, M., Takao, T., Hoshino, M., Shimonishi, Y., Tsuji, S., Begum, N. A., Takeuchi, O., Akira, S., Toyoshima, K. and Seya, T. (2001). *Mycoplasma fermentans* lipoprotein M161Ag-induced cell activation is mediated by Toll-like receptor 2: role of N-terminal hydrophobic portion in its multiple functions. *Journal of Immunology*, 166(4): 2610-2616.

Nolan, M. J., Tomley, F. M., Kaiser, P. and Blake, D. P. (2015). Quantitative real-time PCR (qPCR) for *Eimeria tenella* replication - implications for experimental refinement and animal welfare. *Parasitology International*, 64(5): 464-470.

Norton, C. C. and Hein, H. E. (1976). *Eimeria maxima:* a comparison of two laboratory strains and a fresh isolate. *Parasitology*, 72: 345-354.

Oswald, I. P., Gazzinelli, R., Sher, A. and James, S. L. (1992). IL-10 synergizes with IL-4 and transforming growth factor-beta to inhibit macrophage cytotoxic activity. *Journal of Immunology*, 148(11): 3578-3583.

Oven, I., Resman Rus, K., Dušanić, D., Benčina, D., Keeler, C. L. and Narat, M. (2013). Diacylated lipopeptide from *Mycoplasma synoviae* mediates TLR15 induced innate immune responses. *Veterinary Research*, 44(99): DOI: 10.1186/1297-9716-1144-1199.

Ozaki, K., Spolski, R., Ettinger, R., Kim, H., Wang, G., Qi, C., Hwu, P., Shaffer, D. J., Akilesh, S., Roopenian, D. C., Morse, H. C., Lipsky, P. E. and Leonard, W. J. (2004). Regulation of B cell differentation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. *The Journal of Immunology*, 173(9): 5361-5371.

Ozaki, K., Spolski, R., Feng, C. G., Qi, C. F., Cheng, J., Sher, A., Morse, H. C., Liu, C., Schwartzberg, P. L. and Leonard, W. J. (2002). A critical role for IL-21 in regulating immunoglobulin production. *Science*, 298(5598): 1630-1634.

Paark, H. K., Cho, M. K., Park, H. Y., Kim, K. U., Kim, Y. S., Lee, M. K., Park, S. K., Kim, D. H. and Yu, H. S. (2012). Macrophage migration inhibitory factor isolated from a parasite inhibited Th2 cytokine production in PBMCs of atopic asthma patients. *Immunopathogenesis*, 49(1): 10-15.

Paul, M. S., Paolucci, S., Barjesteh, N., Wood, R. D., Schat, K. A. and Sharif, S. (2012). Characterisation of chicken thrombocyte responses to Toll-like receptor ligands. *PLoS One*, 7(8): doi.org/10.1371/journal.pone.0043381.

Penniall, R. and Spitznagel, J. K. (1975). Chicken neutrophils: oxidative metabolism in phagocytic cells devoid of myeloperoxidase. *Proceedings of the National Academy of Sciences of the United States of America*, 72(12): 5012-5015.

Peranteau, W. H., Zhang, L., Muvarak, N., Badillo, A. T., Radu, A., Zoltick, P. W. and Liechty, K. W. (2008). IL-10 overexpression decreases inflammatory mediators and promotes regenerative healing in an adult model of scar formation. *Journal of Investigative Dermatology*, 128(7): 1852-1860.

Pérez-Mazliah, D., Ng, D. H., Freitas Do Rosário, A. P., Mclaughlin, S., Mastelic-Gavillet, B., Sodenkamp, J., Kushinga, G. and Langhorne, J. (2015). Disruption of IL-21 signalling affecfts T cell-B cell interactions and abrogates protective humoral immunity to malaria. *PloS Pathogens*, 11(3): e1004715.

Petrone, V. M., Constantino, C. F. and Pradal-Roa, P. (2002). Identification and quantification of granulocytes in caecal mucosa and submucosa of chickens experimentally infected with *Eimeria* tenella and *Salmonella enteritidis*. *British Poultry Science*, 43(5): 653-661.

Pickert, G., Neufert, C., Leppkes, M., Zheng, Y., Wittkopf, N., Warntjen, M., Lehr, H. A., Hirth, S., Weigmann, B., Wirtz, S., Ouyang, W., Heurath, M. F. and Becker, C. (2009). STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. *Journal of Experimental Medicine*, 206(7): 1465-1472.

merkle, J., Methner, U. and Berndt, A. (2008). Heterogeneity of avian gammadelta T cells. *Veterinary Immunology and Immunopathology*, 124(3-4): 241-252.

Pierce, A. E., Long, P. L. and Horton-Smith, C. (1963). Attempts to induce a passive immunity to *Eimeria tenella* in young fowls (Gallus domesticus). *Immunology*, 6: 37-47.

Pogonka, T., Schelzke, K., Stange, J., Papadakis, K., Steinfelder, S., Liesenfeld, O. and Lucius, R. (2010). CD8+ cells protect mice against reinfection with the intestinal parasite *Eimeria falciformis*. *Microbes and Infection*, 13(3): 218-226.

Powell, F. L., Rothwell, L., Clarkson, M. J. and Kaiser, P. (2009). The turkey, compared to the chicken, fails to mount an effective early immune response to *Histomonas meleagridis* in the gut. *Parasite Immunology*, 31(6): 312-327.

Quere, P., Cooper, M. D. and Thorbecke, G. J. (1990). Characterisation of suppressor T cells for antibody production by chicken spleen cells. I. Antigen-induced suppressor cells are CT8+, TcR1+ (gamma delta) T cells. *Immunology*, 71(4): 517-522.

Qureshi, M. A. (1998). Role of macrophages in avian health and disease. *Poultry Science*, 77(7): 978-982.

Qureshi, M. A., Marsh, J. A., Dietert, R. R., Sung, Y. J., Nicolas-Bolnet, C. and Petitte, J. N. (1994). Profiles of chicken macrophage effector functions. *Poultry Science*, 73(7): 1027-1034.

Radosevic, K., Rodriguez, A., Lemckert, A. A., Vandermeer, M., Gillissen, G., Warner, C., Voneyben, R., Pau, M. G. and Goudsmit, J. (2010). The Th1 immune response to Plasmodium falciparum circumsporozoite protein is boosted by adenovirus vectors 35 and 26 with a homologous insert. *Clinical and Vaccine Immunology*, 17(11): 1687-1694.

Raffatellu, M., George, M. D., Akiyama, Y., Hornsby, M. J., Nuccio, S. P., Paixao, T. A., Butler, B. P., Chu, H., Santos, R. L., Berger, T., Mak, T. W., Tsolis, R. M., Bevins, C. L., Solnick, J. V., Dandekar, S. and Bäumler, A. J. (2009). Lipcalin-2 resistance confers an advantage to *Salmonella enterica* seroptype *Typhimurium* for growith and survival in the inflamed intestine. *Cell Host Microbe*, 5(5): 476-486.

Rath, N. C., Parcells, M. S., Xie, H. and Santin, E. (2003). Characterisation of a spontaneously transformed chicken mononuclear cell line. *Veterinary Immunology and Immunopathology*, 96(1-2): 93-104.

Rauf, A., Khatri, M., Murgia, M. V. and Saif, Y. M. (2011). Expression of perforin-granzyme pathway genes in the bursa of infectious bursal disease virus-infected chickens. *Developmental and Comparative Immunology*, 35(5): 620-627.

Reed, S. G., Brownell, C. E., Russo, D. M., Silva, J. S., Grabstein, K. H. and Morrissey, P. J. (1994). IL-10 mediates susceptibility to *Trypanosoma cruzi* infection. *1994*, 153(7): 3135-3140.

Roark, C. L., Simonian, P. L., Fontenot, A. P., Born, W. K. and O'brien, R. L. (2008). gammadelta T cells: an important source of IL-17. *Current Opinions in Immunology*, 20(3): 353-357.

Rose, M. E. (1967). Immunity to *Eimeria brunetti* and *Eimeria maxima* infections in the fowl. *Parasitology*, 57(2): 363-370.

Rose, M. E. (1974a). Immunity to four species of *Eimeria* in fowls. *Immunology*, 5(1): 79-82.

Rose, M. E. (1974b). Protective antibodies in infections with *Eimeria maxima*: the reduction of pathogenic effects *in vivo* and a comparison between oral and subcutaneous administration of antiserum. *Parasitology*, 68(3): 285-292.

Rose. M. E. and Hesketh, P. (1976). Immunity to coccidiosis: stages of the life-cycle of *Eimeria maxima* which induce, and are affected by, the response of the host. *Parasitology.* 73: 25-37.

Rose, M. E. and Hesketh, P. (1979). Immunity to coccidiosis: T-lymphocyte- or B-lymphocyte-deficient animals. *Infection and Immunity*, 26(2): 630-637.

Rose, M. E. and Hesketh, P. (1987). *Eimeria tenella*: effects of immunity on sporozoites within the lumen of the small intestine. *Experimental Parasitology*, 63(3): 337-344.

Rose, M. E., Hesketh, P., Rothwell, L. and Gramzinsky, R. A. (1996). T cell receptor gamma delta lymphocutes and *Eimeria vermiformis* infection. *Infection and Immunity*, 64(11): 4854-4858.

Rose, M. E., Hesketh, P. and Wakelin, D. (1992). Immune control of murine coccidiosis: CD4+ and CD8+ T lymphocytes contribute differentially in resistance to primary and secondary infections. *Parasitology*, 105(Pt3): 349-354.

Rose, M. E., Hesketh, P. and Wakelin, D. (1995). Cytotoxic effects of natural killer cells have no significant role in controlling infection with the intracellular protozoon *Eimeria vermiformis*. *Infection and Immunity*, 63(9): 3711-3714.

Rose, M. E. and Lee, D. L. (1977). Interactions *in vitro* between sporozoites of *Eimeria tenella* and host peritoneal exudate cells: electron microscopical observations. *Zeitschrift fur Parasitenkunde*, 54: 1-7.

Rose, M. E. and Long, P. L. (1962). Immunity to four species of *Eimeria* in fowls. *Immunology*, 5: 79-92.

Rose, M. E. and Long, P. L. (1970). Resistance to *Eimeria* infections in the chicken: the effects of thymectomy, bursectomy, whole body irradiation and cortisone treatment. *Parasitology*, 60(2): 291-299.

Rose, M. E., Ogilvie, B. M. and Bradley, J. W. (1980). Intestinal mast cell response in rats and chickens to coccidiosis, with some properties of chicken mast cells. *International Archives of Allergy and Applied Immunology*, 63(1): 21-29.

Rose, M. E., Orlans, E., Payne, A. W. and Hesketh, P. (1981). The origin of IgA in chicken bile: its rapid active transport from blood. *European Journal of Immunology*, 11(7): 561-564.

Rose, M. E., Smith, A. and Wakelin, D. (1991). Gamma interferon-mediated inhibition of *Eimeria vermiformis* growth in cultured fibroblasts and epithelial cells. *Infection and Immunity*, 59(2): 580-586.

Rose, M. E., Wakelin, D. and Hesketh, P. (1989). Gamma interferon controls *Eimeria vermiformis* primary infection in BALB/c mice. *Infection and Immunity*, 57(5): 1599-1603.

Rosenberg, B., Juckett, D. A., Aylsworth, C. F., Dimitrov, N. V., Ho, S. C., Judge, J. W., Kessel, S., Quensen, J., Wong, K. P., Zlatkin, I. and Zlatkin, T. (2005). Protein from intestinal *Eimeria* protozoan stimulated IL-12 release from dendritic cells, exhibits antitumor properties *in vivo* and is correlated with low intestinal tumorigenicity. *International Journal of Cancer*, 114(5): 756-765.

Rosenberg, H. F., Dyer, K. D. and Foster, P. S. (2013). Eosinophils: changing perspectives in health and disease. *Nature Reviews. Immunology*, 13: 9-22.

Rosenberg, M. M., Alicata, J. E. and Palafox, A. L. (1954). Further evidence of hereditary resistance and susceptibility to caecal coccidiosis in chickens. *Poultry Science* 33: 972-980.

Rothwell, C. J., Vervelde, L. and Davison, T. F. (1996). Identification of chicken Bu-1 alloantigens using the monoclonal antibody AV20. *Veterinary Immunology and Immunopathology*, 55(1-3): 225-234.

Rothwell, L., Gramzinski, R. A., Rose, M. E. and Kaiser, P. (1995). Avian coccidiosis: changes in intestinal lymphocyte populations associated with the development of immunity to *Eimeria maxima*. *Parasite Immunology*, 17(10): 525-533.

Rothwell, L., Hu, T., Wu, Z. and Kaiser, P. (2012). Chicken interleukin-21 is costimulatory for T cells and blocks maturation of dendritic cells. *Developmental and Comparative Immunology*, 36(2): 475-482.

Rothwell, L., Muir, W. and Kaiser, P. (2000). Interferon-gamma is expressed in both gut and spleen during *Eimeria tenella* infection. *Avian Pathology*, 29(4): 333-342.

Rothwell, L., Young, J. R., Zoorob, R., Whittaker, C. A., Hesketh, P., Archer, A., Smith, A. and Kaiser, P. (2004). Cloning and characterisation of chicken IL-10 and its role in the immune response to *Eimeria maxima*. *Journal of Immunology*, 173(4): 2675-2682.

Rousset, F., Garcia, E., Defrance, R., Péronne, C., Vezzio, N., Hsu, D. H., Kastelein, R. A., Moore, K. W. and Banchereau, J. (1992). Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. *Proceedings of the National Academy of Sciences of the United States of America*, 89(5): 1890-1893.

Rovedetti, L., Kudo, T., Biancheri, P., Sarra, M., Knowles, C. H., Rampton, D. S., Corazza, G. R., Monteleone, G., Disabatino, A. and Macdonald, T. T. (2009). Differential regulation of interleukin 17 and interferon gamma production in inflammatory bowel disease. *Gut*, 58(12): 1629-1636.

Ruckert, R., Bulfone-Paus, S. and Brandt, K. (2007). Interleukin-21 stimulates antigen uptake, protease activity, survival and induction of CD4 T cell proliferation by murine macrophages. *Clinical and Experimental Immunology*, 151(3): 487-495.

Ruiz, F. M., Fernández, I. S., López-Merino, L., Lagartera, L., Kaltner, H., Menéndez, M., André, S., Solís, D., Gabius, H. J. and Romero, A. (2013). Fine-tuning of prototype chicken galectins: structure of CG-2 and structure-activity correlations. *Biological Crystallography*, 69(Pt9): 1665-1676.

Sand, J. M., Arendt, M. K., Repasy, A., Deniz, G. and Cook, M. E. (2016). Oral antibody to interleukin-10 reduces growth rate depression due to *Eimeria* spp. infection in broiler chickens. *Poultry Science*, 95(2): 439-446.

Sarra, M., Monteleone, I., Stolfi, C., Fantini, M. C., Sileri, P., Sica, G., Tersigni, R., Macdonald, T. T., Pallone, F. and Monteleone, G. (2010). Interferon-gamma-expressing cells are a major source of interleukin-21 in inflammatory bowel diseases. *Inflammatory Bowel Diseases*, 16(8): 1332-1339.

Sato, T., Vries, R. G., Snippert, H. J., Van De Wetering, M., Barker, N., Stange, D. E., Van Es, J. H., Abo, A., Kujala, P., Peters, P. J. and Clevers, H. (2009). Single Lgr5 stem cells build crypt-villus structures without a mesenchymal niche. *Nature*, 459(7244): 262-265.

Schat, K. A., Kaspers, B. and Kaiser, P. (2013). Avian Immunology.

Schild, H., Mavaddat, N., Litzenberger, C., Ehrich, E. W., Davis, M. M., Bluestone, J. A., Matis, L., Draper, R. K. and Chien, Y. H. (1994). The nature of major histocomatibility complex recognition by gamma delta T cells. *Cell*, 76(1): 29-37.

Schmid, M., Heitlinger, E., Spork, S., Mollenkopf, H. J., Lucius, R. and Gupta, N. (2014). *Eimeria falciformis* infeciton of the mouse caecum identifies opposing roles of IFNgamma-related host pathways for the parasite development. *7*, 4(969-982).

Scott, T. and Owens, M. D. (2008). Thrombocytes respond to lipopolysaccharide through Toll-like receptor 4, and MAP kinase and NF-kappaB pathways leading to expression of interleukin-6 and cyclooxygenase-2 with production of prostaglandin E2. *Molecular Immunology*, 45(4): 1001-1008.

Sengupta, N. and Macdonald, T. T. (2007). The role of matrix metalloproteinases in stromal/epithelial interactions in the gut. *Physiology*, 22: 401-409.

Shanmugasundaram, R. and Selvaraj, R. K. (2011). Regulatory T cell properties of chicken CD4+CD25+ cells. *Journal of Immunology*, 186(4): 1997-2002.

Shanmugasundaram, R., Sifri, M. and Selvaraj, R. K. (2013). Effect of yeast cell product (CitriStim) supplementation on broiler performance and intestinal immune cell parameters during an experimental coccidial infeciton. *Poultry Science*, 92(2): 358-363.

Shi, F. and Erf, G. F. (2012). IFN-gamma, IL-21 and IL-10 co-expression in evolving autoimmune vitiligo lesions of Smyth line chickens. *Journal of Investigative Dermatology*, 132(3 Pt 1): 642-649.

Shirley, M. W., Smith, A. L. and Blake, D. P. (2007). Challenges in the successful control of the avian coccidia. *Vaccine*, 25(30): 5540-5547.

Shirley, M. W., Smith, A. L. and Tomley, F. M. (2005). The biology of avian *Eimeria* with an emphasis on their control by vaccination. *Advanced Parasitology*, 60: 285-330.

Shouval, D. S., Biswas, A., Goettel, J. A., Mccann, K., Conaway, E., Redhu, N. S., Mascanfroni, I. D., Adham, Z. A., Lavoie, S., Ibourk, M., Nguyen, D. D., Samsom, J. N., Ebens, J. C., Somech, R., Weiss, B., Beier, R., Conklin, L. S., Ebens, C. L., Santos, F. G.

M. S., Ferreira, A. R., Sherlock, M., Bhan, A. K., Müller, W., Mora, J. R., Quintana, F. J., Klein, C., Muise, A. M., Horwitz, B. H. and Snapper, S. B. (2014). Interleukin-10 receptor signalling in innate immune cells regulates mucosal immune tolerance and anti-inflammatory macrophage function. *Immunity*, 40(5): 706-719.

Smith, A., Hesketh, P., Archer, A. and Shirley, M. W. (2002). Antigenic diversity in *Eimeria maxima* and the influence of host genetics and immunisation schedule on cross-protective immunity. *Infection and Immunity*, 70(5): 2472-2479.

Smith, A. L. and Hayday, A. C. (2000a). An alpha beta T-cell-dependent immunoprotective response towards gut coccidia is supported by gamma delta cells. *Immunology*, 101(3): 325-332.

Smith, A. L. and Hayday, A. C. (2000b). An alpha beta T cell-independent immunoprotective response towards gut coccidia is supported by gamma delta T cells. *Immunology*, 101(3): 325-332.

Smith, A. L., Rose, M. E. and Wakelin, D. (1994). The role of natural killer cells in resistance to coccidiosis: investigations in a murine model. *Clinical and Experimental Immunology*, 97(2273-279).

Smith, N. C., Wallach, M., Petracca, M., Braun, R. and Eckert, J. (1994). Maternal transfer of antibodies induced by infection with *Eimeria maxima* partially protects chickens against challenge with *Eimeria tenella*. *Parasitology*, 109(Pt 5): 551-557.

Smyth, M. J., Wallace, M. E., Nutt, S. L., Yagita, H., Godfrey, D. I. and Hayakawa, Y. (2005). Sequential activation of NKT cells and NK cells provides effective innate immunotherapy of cancer. *The Journal of Experimental Medicine*, 201(12): 1973-1985.

Song, X., Dai, D., She, X., Zhu, S., Yao, Y., Gao, H., Wang, J., Qu, F., Qiu, J., Wang, H., Li, X., Shen, N. and Qiang, Y. (2015). Growth Factor FGF2 Cooperates with Interleukin-17 to Repair Intestinal Epithelial Damage. *Immunity*, 43(3): 488-501.

Spolski, R., Kim, H., Zhu, W., Levy, D. E. and Leonard, W. J. (2009). IL-21 mediates suppressive effects via its induction of IL-10. *Journal of immunology*, 182(5): 2859-2867.

Staines, K., Hunt, L. G., Young, J. R. and Butter, C. (2014). Evoluiton of an expanded mannose receptor gene family. *PLoS One*, 9(11): e110330.

Stange, J., Hepworth, M. R., Rausch, S., Zajic, L., Kühl, A. A., Uyttenhove, C., Renauld, J. C., Hartmann, S. and Lucius, R. (2012). IL-22 mediates host defense against an intestinal intracellular parasite in the absense of IFN-gamma at the cost of Th17-driven immunopathology. *Journal of Immunology*, 188(5): 2410-2418.

Stone, K. D., Prussin, C. and Metcalfe, D. D. (2010). IgE, mast cells, basophils, and eosinophils. *Journal of Allergy and Clinical Immunology*, 125(2): 73-80.

Strengell, M., Saraneva, T., Foster, D., Julkunen, I. and Matikainen, S. (2002). IL-21 up-regulates the expression of geens associated with innate immunity and Th1 response. *Journal of Immunology*, 169(7): 3600-3605.

Stritesky, G. L., Yeh, N. and Kaplan, M. H. (2008). IL-23 promotes maintenance but not commitment to the Th17 lineage. *Journal of Immunology*, 181(9): 5948-5955.

Stumhofer, J. S., Silver, J. S. and Hunter, C. A. (2013a). IL-21 is required for optimal antibody productin and T cell responses during chronic *Toxoplasma gondii* infection. *PLoS One*, 8(5): e62889.

Stumhofer, J. S., Silver, J. S. and Hunter, C. A. (2013b). IL-21 is required for optimal antibody production and T cell responses during chronic *T. gondii* infection. *PLoS One*, 8(5).

Sung, Y. J., Hotchkiss, J. H., Austic, R. E. and Dietert, R. R. (1991). L-arginine-dependent production of a reactive nitrogen intermediate by macrophages of a uricotelic species. *Journal of Leukocyte Biology*, 50(1): 49-56.

Sutherland, A. P. R., Joller, N., Michaud, M., Liu, S. M., Kuchroo, V. J. and Grusby, M. J. (2013). IL-21 promotes CD8+ CTL activity via the transcription factor T-bet. *Journal of Immunology*, 190(8): 3977-3984.

Suto, A., Wurster, A. L., Reiner, S. L. and Grusby, M. J. (2006). IL-21 inhibits IFN-gamma production in developing Th1 cells through the repression of Eomesodermin expression. *Journal of Immunology*, 177(6): 3721-3727.

Sutton, K. (2014). The functions of receptor activator of NF-kappaB ligand (RANKL) and its receptors, RANK and OPG, are evolutionarily conserved. (Doctoral thesis, University of Edinburgh, UK). Retrieved from https://www.era.lib.ed.ac.uk/bitstream/handle/1842/10041/Sutton2014.pdf?sequence=2&isAllowed=y.

Swaggerty, C. L., Genovese, K. J., He, H., Duke, S. E., Pevzner, I. Y. and Kogut, M. H. (2011). Broiler breeders with an efficient innate immune response are more resistant to *Eimeria tenella*. *Poultry Science*, 90(5): 1014-1019.

Swaggerty, C. L., Pevzner, I. Y. and Kogut, M. H. (2015). Selection for pro-inflammatory mediators produces chickens more resistant to *E. tenella Poultry Science*, 94(1): 37-42.

Swihart, K., Fruth, U., Messmer, N., Hug, K., Behrin, R., Huang, S., Del Giudice, G., Aguet, M. and Louis, J. A. (1995). Mice from a genetically resistant background lacking the interferon gamma receptor are susceptible to infection with Leishmania major but mount a polarized T helper cell 1-type CD4+ T cell response. *Journal of Experimental Medicine*, 181(3): 961-971.

Swinkels, W. J. (2008). Host response to *Eimeria* infection (Doctoral thesis, Utrecht University, Netherlands). Retrieved from https://dspace.library.uu.nl/bitstream/handle/1874/27665/swinkels.pdf?sequence=2.

Swinkels, W. J., Post, J., Cornelissen, J. B. W. J., Engel, B., Boersma, W. J. A. and Rebel, J. M. J. (2006). Immune responses in *Eimeria acervulina* infected one-day-old broilers compared to amount of *Eimeria* in the duodenum, measured by real-time PCR. *Veterinary Parasitology*, 138(3-4): 223-233.

Swinkels, W. J., Post, J., Cornelissen, J. B. W. J., Engel, B., Boersma, W. J. A. and Rebel, J. M. J. (2007). Immune responses to an *Eimeria acervulina* infection in different broiler lines. *Veterinary Immunology and Immunopathology*, 117(1-2): 26-34.

Taubert, A., Behrendt, J., Sühwold, A., Zahner, H. and Hermosilla, C. (2009). Monocyte- and macrophage-mediated immune reactions against *Eimeria bovis*. *Veterinary Parasitology*, 164(141-153).

Tesmer, L. A., Lundy, S. K., Sarkar, S. and Fox, D. A. (2008). Th17 cells in human disease. *Immunological Reviews*, 223: 87-113.

Thelemann, C., Eren, R. O., Coutaz, M., Brasseit, J., Bouzourene, H., Rosa, M., Duval, A., Lavanchy, C., Mack, V., Mueller, C., Reith, W. and Acha-Orbea, H. (2014). Interferon-gamma induces expression of MHC class II on intestinal epithelial cells and protects mice from colitis. *PLoS One*, 9(1): DOI: 10.1371/journal.pone.0086844.

Thompson-Snipes, L., Dhar, V., Bond, M. W., Mosmann, T. R., Moore, K. W. and Rennick, D. (1991). Interleukin 10: a novel stimulatory factor for mast cells and their progenitors. *Journal of Experimental Medicine*, 173(2): 507-510.

Thorbecke, G. J., Gordon, H. A., Wostman, B., Wagner, M. and Revniers, J. A. (1957). Lymphoid tissue and serum gamma globulin in young germ free chickens. *Journal of Infectious Diseases*, 101(3): 237-251.

Trees, A. J., Karim, M. J., Mckellar, S. B. and Carter, S. D. (1989). *Eimeria tenella*: local antibodies and interactions with the sporozoite surface. *Journal of Protozoology*, 36(4): 326-333.

Tregaskes, C. A., Bumstead, N., Davison, T. F. and Young, J. R. (1996). Chicken B-cell marker chB6 (Bu-1) is a highly glycosylated protein of unique structure. *Immunogenetics*, 44(3): 212-217.

Tregaskes, C. A., Glansbeek, H. L., Gill, A. C., Hunt, L. G., Burnside, J. and Young, J. R. (2005). Conservation of biological properties of the CD40 ligand, CD154 in a non-mammalian vertebrate. *Developmental and Comparative Immunology*, 29(4): 361-374.

Trout, J. M. and Lillehoj, H. S. (1996). T lymphocyte roles during *Eimeria acervulina* and *Eimeria tenella* infections. *Veterinary Immunology and Immunopathology*, 53(1-2): 163-172.

Urban, J. F., Fayer, R., Chen, S. J., Gause, M. K., Gately, M. K. and Finkelman, F. D. (1996). IL-12 protects immunocompetent and immunodeficient neonatal mice against infection with *Cryptosporidium parvum*. *Journal of Immunology*, 156(1): 263-268.

Vainio, O., Koch, G. and Toivanen, A. (1984). B-L antigens (class II) of the chicken major histocompatibility complex control T-B cell interaction. *Immunogenetics*, 19(2): 131-140.

Velkers, F. C., Swinkels, W. J. C., Rebel, J. M. J., Bouma, A., Daemen, A. J. J. M., Klinkenberg, D., Boersma, W. J. A., Stegeman, J. A., Dejong, M. C. M. and Heesterbeek, J. a. P. (2010). Effect of *Eimeria acervulina* infection history on the immune response and transmission in broilers. *Veterinary Parasitology*, 173: 184-192.

Vervelde, L. and Jeurissen, S. H. M. (1993a). Postnatal development of intra-epithelial leukocytes in the chicken digestive tract: phenotypical characterisation *in situ*. *Cell and Tissue Research*, 274(2): 295-301.

Vervelde, L., Vermeulen, A. N. and Jeurissen, S. H. (1993b). Common epitopes on *Eimeria tenella* sporozoites and caecal epithelium of chickens. *Infection and Immunity*, 61(10): 4504-4506.

Vervelde, L., Vermeulen, A. N. and Jeurissen, S. H. (1995). *Eimeria tenella:* sporozoites rarely enter leukocytes in teh caecal epithelium of the chicken (*Gallus domesticus*). *Experimental Parasitology*, 81(1): 29-38.

Vervelde, L., Vermeulen, A. N. and Jeurissen, S. H. (1996). *In situ* characterisation of leukocyte subpopulations after infection with *Eimeria tenella* in chickens. *Parasite Immunology*, 18(5): 247-256.

Vetterling, J. M. and Doran, D. J. (1966). Schizogony and gametogony in the life cycle of the poultry coccidium, *Eimeria acervulina* Tyzzer, 1929. *Journal of Parasitology*, 52(6): 1150-1157.

Vogelzang, A., Mcguire, H. M., Yu, D., Sprent, J., Mackay, C. R. and King, C. (2008). A fundamental role for interleukin-21 in the generation of T follicular helper cells. *Immunity*, 29(1): 127-137.

Volpini, L. M., Calnek, B. W., Sekellick, M. J. and Marcus, P. I. (1995). Stages of Marek's disease virus latency defined by variable sensitivity to interferon modulation of viral antigen expression. *Veterinary Microbiology*, 47(1-2): 99-109.

Wachowicz, B., Krajewski, T. and Stefanczyk, B. (1981). Antiheparin proteins secreted by avian thrombocytes. *Thrombosis and Haemostasis*, 45(1): 98.

Wakelin, D., Rose, M. E., Hesketh, P., Else, K. J. and Grencis, R. K. (1993). Immunity to coccidiosis: genetic influences on lymphocyte and cytokine responses to infection with *Eimeria vermiformis* in inbred mice. *Parasite Immunology*, 15(1): 11-19.

Walliser, I. and Göbel, T. C. (2017). Generation of glycosylphosphatidylinositol linked chicken IL-17 to generate specific monoclonal antibodies applicable for intracellular cytokine staining. *Developmental and Comparative Immunology*, 73: 27-35.

Wang, L., Li, J., Li, J. J., Li, R. X., Lv, C. F., Li, S., Mi, Y. L. and Zhang, C. Q. (2016). Identification of the Paneth cells in chicken small intestine. *Poultry Science*, 95(7): 1631-1635.

Wang, Y., Jiang, X., Zhu, J., Yue, D., Zhang, X., Wang, X., You, Y., Wang, Y. X., Lu, C., Sun, X. and Yoshikai, Y. (2016). IL-21/IL-21R signalling suppresses intestinal inflammation induced by DSS through regulation of Th responses in lamina propria in mice. *Nature*, 6: doi: 10.1038/srep31881.

Wang, Z., Zhou, S., Sun, C., Lei, T., Peng, J., Li, W., Ding, P., Lu, J. and Zhao, Y. (2015). Interferon-γ inhibits nonopsonized phagocytosis of macrophages via an mTORC1-c/EBPβ pathway. *Journal of Innate Immunity*, 7(2): 165-176.

Wang, Z. E., Reiner, S. L., Zheng, S., Dalton, D. K. and Locksley, R. M. (1994). CD4+ effector cells default to the Th2 pathway in interferon gamma-deficient mice infected with Leishmania major. *Journal of Experimental Medicine*, 179(4): 1367-1371.

Warren, E. W. and Ball, S. J. (1967). Schizogonous stages of *Eimeria acervulina* Tyzzer, 1929. *Nature*, 214(5090): 829-830.

Wattrang, E., Magnussen, S. E., Näslund, K., Thebo, P., Hagström, A., Smith, A. L. and Lundén, A. (2016a). Expression of perforin, granzyme A and Fas ligand mRNA in caecal tissues upon *Eimeria tenella* infection of naive and immune chickens. *Parasite Immunology*, 38(7): 419-430.

Wattrang, E., Thebo, P., Lundén, A. and Dalgaard, T. S. (2016b). Monitoring of local CD8β-expressing cell populations during Eimeria tenella infection of naïve and immune chickens. *Parasite Immunology*, 38(8): 453-467.

Wei, L., Laurence, A., Elias, K. M. and O'shea, J. J. (2007). IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. *The Journal of Biological Chemistry*, 282(48): 34605-34610.

Welch, L. (2015). Th17 immune responses in the chicken (Doctoral thesis, University of Edinburgh, UK). Retrieved from https://www.era.lib.ed.ac.uk/handle/1842/15909.

Wells, L. L., Lowry, V., Deloach, J. R. and Kogut, M. H. (1998). Age-dependent phagocytosis and bactericidal activities of the chicken heterophil. *Developmental and Comparative Immunology*, 22(1): 103-109.

Whyte, S. M., Sylvester, I. D., Martin, S. R., Gill, A. C., Wopfner, F., Schätzl, H. M., Dodson, G. G. and Bayley, P. M. (2003). Stability and conformational properties of doppel, a prion-like protein and its single-disulphide mutant. *Biochemical Journal*, 373(Pt2): 485-494.

Wight, P. a. L. (1970). The mast cells of *Gallas domesticus*. *Acta Anatomica*, 75: 100-113.

Wilson, E. H., Willie-Reece, U., Dzierszinski, F. and Hunter, C. A. (2005). A critical role for IL-10 in limiting inflammation during toxoplasmic encephalitis. *Journal of Neuroimmunology*, 165(1-2): 63-74.

Wu, Z., Hu, T. and Kaiser, P. (2011). Chicken CCR6 and CCR7 are markers for immature and mature dendritic cells respectively. *Developmental and Comparative Immunology*, 35(5): 563-567.

Wu, Z., Hu, T., Rothwell, L., Vervelde, L., Kaiser, P., Boulton, K., Nolan, M. J., Tomley, F. M., Blake, D. P. and Hume, D. A. (2016). Analysis of the function of IL-10 in chickens using specific neutralising antibodies and a sensitive capture ELISA. *Developmental and Comparative Immunology*, 63: 206-212.

Wu, Z., Rothwell, L., Hu, T. and Kaiser, P. (2009). Chicken CD14, unlike mammalian CD14, is trans-membrane rather than GPI-anchored. *Developmental and Comparative Immunology*, 33(1): 97-104.

Wu, Z., Rothwell, L., Young, J. R., Kaufman, J., Butter, C. and Kaiser, P. (2010). Generation and characterisation of chicken bone marrow-derived dendritic cells. *Immunology*, 129(1): 133-145.

Wurster, A. L., Rodgers, V. L., Satoskar, A. R., Whitters, M. J., Young, D. A., Collins, M. and Grusby, M. J. (2002). Interleukin 21 is a T helper (Th) cell 2 cytokine that specifically inhibits the differentiations of naive Th cells into interferon gamma-producing Th1 cells. *Journal of Experimental Medicine*, 196(7): 969-977.

Xing, Z. and Schat, K. A. (2000). Expression of cytokine genes in Marek's disease virus-infected chickens and chicken embryo fibroblast cultures. *Immunology*, 100(1): 70-76.

Xu, X., Weiss, I. D., Zhang, H. H., Singh, S. P., Wynn, T. A., Wilson, M. S. and Farber, J. M. (2014). Conventional NK cells can produce IL-22 and promote host defense in Klebsiella pneumoniae pneumonia. *Journal of Immunology*, 192(4): 1778-1786.

Yadav, A. and Gupta, S. K. (2001). Study of resistance against some ionophores in *Eimeria tenella* field isolates. *Veterinary Parasitology*, 102(1-2): 69-75.

Yarovinsky, F., Zhang, D., Anderson, J. F., Bannenberg, G. L., Serhan, C. N., Hayden, M. S., Hieny, S., Sutterwala, F. S., Flavell, R. A., Ghosh, S. and Sher, A. (2005). TLR11 activation of dendritic cells by a protozoan profilin-like protein. *Science*, 308(5728): 1626-1629.

Yasuda, M., Taura, Y., Yokomizo, Y. and Ekino, S. (1998). A comparative study of germinal centre: fowls and mammals. *Comparative Immunology, Microbiology and Infectious Disease*, 21(3): 179-189.

Yen, D., Cheung, J., Scheerens, H., Poulet, F., Mcclanahan, T., Mckenzie, B., Kleinschek, M. A., Owyang, A., Mattson, J., Blumenschein, W., Murphy, E., Sathe, M., Cua, D. J., Kastelein, R. A. and Rennick, D. (2006). IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *Journal of Clinical Investigation*, 116(5): 1310-1316.

Yi, J. S., Du, M. and Zajac, A. J. (2009). A vital role for interleukin-21 in the control of a chronis viral infection. *Science*, 324(5934): 1572-1576.

Yin, G., Qin, M., Liu, X., Suo, J., Tang, W., Tao, G., Han, Q., Suo, X. and Wu, W. (2013). An *Eimeria* vaccine candidate based on *Eimeria tenella* immune mapped protein 1 and the TLR5 agonist *Salmonella tyhphiumurium* FliC flagellin. *Biochemical and Biophysical Research Communications*, 440(3): doi: 10.1016/j.bbrc.2013.1009.1088.

Young, J. R., Davison, F., Tresgaskes, C. A., Rennie, M. C. and Vainio, O. (1994). Monomeric homologue of mammalian CD28 is expressed on chicken T cells. *Journal of Immunology*, 152(8): 3848-3851.

Yun, C. H., Lillehoj, H. S. and Choi, K. D. (2000). *Eimeria tenella* infectiton induces local gamma interferon production and intestinal lymphocyte subpopulation changes. *Infection and Immunity*, 68(3): 1282-1288.

Zeng, R., Spolski, R., Finkelstein, S. E., Oh, S., Kovanen, P. E., Hinrichs, C. S., Pise-Masison, C. A., Radonovich, M. F., Brady, J. N., Restifo, N. P., Berzofsky, J. A. and Leonard, W. J. (2005). Synergy of IL-21 and IL-15 in regulating CD8 T cell expansion and function. *The Journal of Experimental Medicine*, 201(1): 139-148.

Zhang, H., Xia, B., Li, J., Zhao, Q., Chen, Z. T., Zhou, R. and Wu, J. (2016). Expression and clinical significance of IL-17 and IL-17 receptor in ulcerative colitis. *Journal of Huazhong University of Science and Technology*, 36(1): 37-40.

Zhang, L., Liu, R., Ma, L., Wang, Y., Pan, B., Cai, J. and Wang, M. (2012). *Eimeria tenella*: Expression profiling of toll-like receptors and associated cytokines in the caecum of infected day-old and three-week old SPF chickens. *Experimental Parasitology*, 130: 442-448.

Zhang, L., Liu, R., Song, M., Hu, Y., Pan, B., Cai, J. and Wang, M. (2013). *Eimeria tenella*: interleukin 17 contributes to host immunopathology in the gut during experimental infection. *Experimental Parasitology*, 133(2): 121-130.

Zhang, L., Lu, L., Li, S., Zhang, G., Ouyang, L., Robinson, K., Tang, Y., Zhu, Q., Li, D., Hu, Y. and Liu, Y. C. (2016). 1,25-Dihydroxyvitamin-D3 induces avian beta-defensin gene expression in chickens. *PLoS One*, 11(5): doi: 10.1371/journal.pone.0154546.

Zhang, Q., Eicher, S. and Applegate, T. J. (2015). Development of intestinal mucin 2, IgA and polymeric Ig receptor expressions in broiler chickens and Pekin ducks. *Poultry Science*, 94(2): 172-180.

Zhao, C., Nguyen, T., Liu, L., Sacco, R. E., Brogden, K. A. and Lehrer, R. I. (2001). Gallinacin-3, an inducible epithelial beta-defensin in the chicken. *Infection and Immunity*, 69(4): 2684-2691.

Zheng, Y., Valdez, P. A., Danilenko, D. M., Hu, Y., Sa, S. M., Gong, Q., Abbas, A. R., Modrusan, Z., Ghilardi, N., Desauvage, F. J. and Ouyang, W. (2008). Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nature Medicine*, 14(3): 282-289.

Zhou, Z., Wang, Z., Cao, L., Hu, S., Zhang, Z., Qin, B., Guo, Z. and Nie, K. (2013). Upregulation of chicken TLR4, TLR15 and MyD88 in heterophils and monocyte-derived macrophages stimulated with *Eimeria tenella in vitro*. *Experimental Parasitology*, 133: 427-433.

Zhu, J., Krishnegowda, G., Li, G. and Gowda, D. C. (2011). Proinflammatory responses by glycosylphosphatidylinositols (GPIs) of *Plasmodium falciparum* are mainly mediated through the recognition of TLR2/TLR1. *Experimental Parasitology*, 128(3): 205-211.

Zhu, Q. and Kanneganti (2017). Cutting edge: Distinct regulatory mechanisms control proinflammatory cytokines IL-18 and IL-1B. *Journal of Immunology*: doi.org/10.4049/jimmunol.1700352.

Ziesché, E., Bachmann, M., Kleinhart, H., Pfeilschifter, J. and Mühl, H. (2007). The interleukin-22/STAT3 pathway potentiates expression of inducible nitric oxide synthase in human colon carcinoma cells. *Journal of Biological Chemistry*, 282(22): 16006-16015.

Zigterman, G. J., Van De Ven, W., Van Geffen, C., Loeffen, A. H., Panhuijzen, J. H., Rijke, E. O. and Vermeulen, A. N. (1993). Detection of mucosal immune responses in chickens after immunisation or infection. *Veterinary Immunology and Immunopathology*, 36(3): 281-291.